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DEVELOPING A REFERENCE MATERIAL FOR WHEAT GLUTEN QUANTIFICATION

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ABSTRACTS

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Tiivistelmä/Referat – Abstract

The literature part of the study reviewed the recommended gluten quantification method, immunological ELISA R5. R5 is a monoclonal antibody that recognizes mainly the epitope that is abundant in especially gluten protein subgroup, ω-gliadin. The current PWG-gliadin reference material used in ELISA leads to inaccuracy of the gluten content, because it cannot represent sample materials that differ in their gliadin composition.

The aim of the experimental study was to compare the prolamin compositions of different wheat cultivars and their reactivity against R5 antibody in sandwich ELISA. The aim was to find the most suitable ratio of barley prolamin, C-hordein, to be used as a reference material for wheat gluten quantification. The ω-gliadin proportions of different cultivars were calculated from RP-HPLC-chromatograms. In order to compare the total wheat gluten reactivity of the cultivars in ELISA R5 with gliadin standard and C-hordein in different ratios (10, 20 and 30% in BSA), Km-values that measure the rate of sensitivity in the assay, were calculated.

The method to separate gliadin- and glutenin subgroups in RP-HPLC was optimized (solvent to extract gliadin and glutenin, temperature, injection volume, gradient). For cv. Crusoe the ω-, α/β- and γ-gliadins and HMW- and LMW-glutenins were identified. The selected wheat cultivars were categorized into four groups. The proportion of ω-gliadin in total gliadin ranged from 0.8 to 14.1% between the cultivars, whereas for PWG-gliadin this has been reported to be 7.7%. In terms of similar reactivity (Km-value) in ELISA, 20% C-hordein was found to be the most suitable reference material (Km 90) for the selected wheat cultivars (Km average 92), instead of the current gliadin standard (Km 68). The advantage of C-hordein standard is that the concentration and thus reactivity can be adjusted to match the sample materials with different prolamin profiles. Unlike with current gliadin reference material, it can be used without any conversion factors, which improves the method accuracy.

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Tiivistelmä/Referat – Abstract

Kirjallisuuskatsauksessa käsiteltiin immunologista ELISA R5-metodia, jota käytetään gluteenin määrittämiseen elintarvikkeissa. R5 on monoklonaalinen vasta-aine, joka tunnistaa erityisen hyvin gluteenin fraktion, ω-gliadiinin. ELISA:n nykyinen PWG-gliadiini-referenssimateriaali antaa epätarkkoja tuloksia näytteille, jotka ovat gliadiinikoostumukseltaan poikkeavia.

Kokeellisen osuuden tavoitteena oli vertailla tutkimukseen valittujen vehnälajikkeiden prolamiinikoostumuksia sekä reaktiivisuutta ELISA:ssa. Tavoitteena oli tutkia, millä suhteella ohran prolamiinista, C-hordeiinista, ja BSA:sta saataisiin valituille vehnälajikkeille optimaalisin referenssimateriaali. Tutkimuksen vehnälajikkeiden ω-gliadiinipitoisuudet kokonaisgliadiinista laskettiin HPLC-kromatogrammeista. Lajikkeiden kokonaisgluteenin reaktiivisuutta ELISA R5:ssa vertailtiin gliadiinistandardiin ja C-hordeiinin eri pitoisuuksiin (10, 20 ja 30% BSA:ssa) laskemalla näytteen reaktiivisuutta mittaavat Km-arvot.

Metodi eri gliadiini- ja gluteniiniryhmien erottamiseen RP-HPLC:ssä optimoitiin (liuos gliadiinin ja gluteniinin uuttoon, lämpötila, injektiomäärä, liuottimen pitoisuus ajoliuoksessa). Lajikkeen Crusoe ω-, α/β- ja γ-gliadiinit sekä HMW- ja LMW-gluteniinit identifioitiin. Valitut vehnälajikkeet luokiteltiin neljään ryhmään niiden RP-HPLC-gliadiiniprofiilien mukaisesti. Lajikkeiden ω-gliadiinipitoisuudet vaihtelivat 0.8-14.1 % kokonaisgliadiinista, kun taas PWG-gliadiinille tämän on ilmoitettu olevan 7.7%. Samankaltaisten Km-arvojen perusteella 20% C-hordeiini (Km 90) oli valituille vehnälajikkeille (Km keskiarvo 92) sopivin referenssimateriaali nykyisen gliadiinistandardin (Km 68) sijasta. C-hordeiinin käyttöä referenssimateriaalina puoltaa se, että sen pitoisuutta ja täten reaktiivisuutta ELISA:ssa on mahdollista säätää vastaamaan näytemateriaalia ja täten saavuttaa tarkempia tuloksia gluteenipitoisuudelle kuin nykyisellä gliadiinistandardilla. Toisin kuin gliadiinistandardi, C-hordeiini ei tarvitse kerrointa gluteenipitoisuuden laskemiseen.

Avainsanat – Nyckelord – Keywords

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PREFACE

The experimental part of this Master's thesis was conducted in the Department of Food and Nutrition in University of Helsinki between February and June 2017. The planning of the project started already in the end of year 2016 and the writing of the thesis was finalized in March 2019. The supervisors were postdoctoral researcher Xin Huang and university lecturer Tuula Sontag-Strohm.

It took far more time for me to finish this thesis than I had planned in the beginning. However, while writing this I also finished some courses, got a job and gave birth. In addition to learning so much about gluten analytics, I also have learned to multitask and schedule my days in order to get everything done. It can be concluded that the past two years have been somewhat busy.

I wish to thank my supervisors for their help throughout this project. A big thank you to Tuula for encouragement and advice. Thank you Xin for all your expertise and patience to answer all my questions. I am also grateful for my family and friends for helping me rest and for offering babysitting help. Last but certainly not least, thank you Tuomas for always believing in me and supporting me, and Aatos for helping me to remember what is important.

Sara Leinonen

Helsinki, 29.3.2019

LIST OF ABBREVIATIONS

ACN	acetonitrile
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
HLA	human leukocyte antigen
HMW	high molecular weight
HMW-GS	high molecular weight glutenin subunit
HRP	horse radish peroxidase
LMW	low molecular weight
LMW-GS	low molecular weight subunit
MMW	medium molecular weight
PWG	Prolamin Working Group
RP-HPLC	reversed-phase high-performance liquid chromatography
SDS-PAGE	sodium dodecyl sulphate-polyacryl amide gel electrophoresis
TFA	trifluoroacetic acid

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1 INTRODUCTION

In order that it would be easier for persons intolerant to gluten follow a safe diet, Codex Alimentarius Commission has defined ”standard for special dietary use for persons intolerant to gluten”. According to this standard foods can be labeled gluten-free if the gluten level does not exceed 20 mg/kg in total, based on the food as distributed to the consumer (CAC 2008). CAC recommends enzyme-linked immunosorbent assay (ELISA) R5 method to be used in gluten quantification.

The ELISA R5 sandwich method is able to recognize coeliac-toxic epitope, QQPFP, which occurs in α/β-, γ- and ω-gliadins of wheat, hordeins of barley and secalins of rye (Valdés et al. 2003). Ideally R5 detects gliadins from wheat gluten, and gliadin content of gluten is theoretically taken as 50 %, thus a factor of two is used to calculate the final gluten content (CAC 2008). At the end of the method a value of gluten, expressed in mg/kg or ppm, is obtained (Diaz-Amigo and Popping 2013). This value is the basis for the decision if a product can be labeled gluten-free.

R5 is a monoclonal antibody that recognizes mainly the epitope QQPFP, which occurs in the repetitive domains of prolamins (Osman et al. 2001). R5 recognizes also the epitopes LQPFP, GLPYP, QQSFP, QQTFP, PQPFPF, QQPYP and PQPFP, but to a weaker degree. At the moment the most recommended reference material for ELISA R5 is a gliadin from Prolamin Working Group (PWG) (van Eckert et al. 2006). PWG-gliadin has been extracted from a mixture of European wheat cultivars. The prolamin working group (PWG) gliadin standard is not reproducible and it is not accepted as a certified reference material in the Institute of Reference Material and Measurements of the European Commission due to its high glutenin content (Working Group on Prolamin Analysis and Toxicity 2016).

Currently there can be found over 20 different ELISA kits for gluten analysis (Scherf and Poms 2016). Despite an improvement of extraction methods and the development of new specific antibodies, many challenges still remain with ELISAs. The food matrix and processing history influences and there is variability of results between different ELISA kits. In a study of Ellis et al. (2016) it was found that many products marketed as gluten-free contained considerable quantities of gluten, even those based on naturally gluten-free ingredients only. Also the use of the factor two in the end of the method is problematic, since it is only based on the assumption that the ratio of

gliadin and glutenin is 1 (CAC 2008). R5-, G12- and Skerritt-antibodies are used mainly to analyse wheat prolamins (gliadin), but there is not enough knowledge about their reactivity against wheat glutelins (glutenin) (Diaz-Amigo and Popping 2013).

Based on scientific evidence, new approved reference materials are needed for gluten quantification to ensure the reliability of the analytical methods and thus improve food safety of the gluten-free products. The underestimation of gluten contents represents a serious health risk for gluten intolerant persons and the overestimation unnecessarily limits the availability of gluten-free products (Lexhaller et al. 2016).

In a previous study by Huang et al. (2017) the concentration of barley prolamin (hordein) in gluten-free products was overestimated 1.8-2.5 times by the R5 ELISA method when calibrated against the wheat gliadin standard. One reason for the overestimation may be the different composition of gliadin reference material from that of hordeins. Estimates in the correct range were obtained when the standard was 40 % C-hordein. Based on this study, C-hordein could be possible, more accurate reference material also for wheat gluten quantification. C-hordein is a better reference material than PWG-gliadin, because its repetitive sequences correspond to R5 epitopes (Huang et al. 2017). In addition, the use of C-hordein as calibrant has the possibility of adjustment by the percentage of C-hordein.

The aim of this study was to compare the prolamin compositions of different wheat cultivars and their reactivity against R5 antibody in sandwich ELISA. The aim was to isolate total gluten and to find the most suitable ratio of C-hordein to be used as a reference material for wheat gluten quantification of the selected cultivars instead of current gliadin standard.

2 LITERATURE REVIEW

2.1 Coeliac disease

Coeliac disease is a gluten-sensitive enteropathy that occurs in genetically predisposed persons (Sollid 2002; Ludvigsson et al. 2013). Coeliac disease leads to the destruction of the microscopic projections of the small intestine that are called villi. The disease is triggered by the ingestion of the gluten proteins contained in wheat, barley and rye (Kagnoff 2007). Oats can be tolerated by most gluten intolerant individuals (Haboubi et al. 2006). Epidemiologic studies have shown that coeliac disease affects almost 1% of the world's population (Lee et al. 2006). It is diagnosed worldwide, also in developing countries. Treatment with a strict gluten-free diet is the usual therapy for coeliac disease patients (Morón et al. 2008). However, cereal proteins are widely used additives in most sectors of the prepared-food industry, making the avoidance of gluten difficult.

Disease pathogenesis involves complex interactions among environmental, genetic and immunological factors, which make it difficult to identify the underlying mechanisms of coeliac disease development (Sollid 2002; Kagnoff 2007). The genetic factor that affect coeliac disease is the expression of human leukocyte antigens HLA-DQ2 and HLA-DQ8 (Sollid et al. 1989). More than 90% of coeliac disease patients express the HLA-DQ2 molecule, whereas DQ8 is carried by most of the remaining patients (Bergseng et al. 2008). DQ2- and DQ8-mediated presentation of gluten peptides to $CD4^+$T cells is central event in the pathogenesis of the disease. HLA class II molecules, like DQ2.5 and DQ8, are receptors expressed on the surface of antigen-presenting cells that bind peptide fragments to $CD4^+$T cells (Sollid 2002).

The toxic components of wheat gluten belong to a family closely related proline- and glutamine-rich proteins called gliadins (Shan et al. 2002). It has been shown by in vitro and in vivo studies in rats and humans that a 33-mer peptide from α-gliadin is stable toward breakdown by all gastric, pancreatic, and intestinal microvilli enzymes. This peptide has been identified as the primary initiator of the inflammatory response to gluten in persons with coeliac disease. In a study by Shan et al. (2002) homologs of the 33-mer peptide LQLQPF$(PQPQLPY)_3$PQPQPF were found in all food grains that are toxic to coeliac disease patients (except oats), but they were absent in all nontoxic food grains. The peptide reacted with tissue transglutaminase, which is the major

autoantigen in coeliac disease, with substantially greater selectivity than known natural substrates of this enzyme. It was a potent inducer of gut-derived human T cell lines from 14 of 14 coeliac disease patients. The 33-mer peptide could be detoxified in in vitro and in vivo assays by exposure to a bacterial prolyl endopeptidase (Shan et al. 2002). This could be a strategy for oral peptidase supplement therapy for coeliac disease.

2.2 Gluten classification and antibody epitopes

2.2.1 Cereal prolamins

Prolamins are plant storage proteins that are insoluble in water or aqueous salt solutions in their native state (Shewry et al. 1999). Prolamins of wheat are called gliadins and glutenins, of rye secalins, of barley hordeins and of oat avenins (Shewry and Tatham 1999). Wheat, rye and barley are classified in the same tribe (the Triticeae), whereas oat is classified in the tribe Aveneae.

Prolamins can be classified into various groups and sub-groups based on their structural relationships. The classification of prolamins from wheat, barley and rye is shown in table 1. There are three different groups of prolamins in Triticeae; the sulphur-rich, sulphur-poor and high molecular weight (HMW) prolamins (Shewry and Tatham 1999). Monomeric prolamins are soluble in aqueous alcohol solutions in their native state and polymeric prolamins are alcohol-soluble when reduced by reductive agents or hydrolysed by enzymes (Shewry et al. 1999). Monomeric and polymeric prolamins have intrachain disulphide bonds, but in addition polymeric prolamins have interchain disulphide bonds (Wieser 2007). For wheat, prolamins form the major storage protein fraction (Shewry and Tatham 1999).

Table 1. Classification of prolamins proteins from wheat, barley and rye.

	HMW prolamins	**Sulphur-rich prolamins**	**Sulphur-poor prolamins**
Wheat	HMW-glutenin subunits (x-types) HMW-glutenin subunits (y-types)	γ-gliadins α/ β-gliadins LMW-glutenins	ω-gliadins
Barley	D-hordeins	γ-hordeins B-hordeins	C-hordeins
Rye	HMW-secalins	γ-secalins	ω-secalins

2.2.2 Gliadins

Wheat gluten consists of gliadin and glutenin fractions which are present in approximately equal amounts (Shewry 2003). The characterization of gliadins and glutenins is shown in table 2. Gliadins are mainly monomeric proteins with molecular weights (MW) varying from 28000 to 55000 (Wieser 2007). Based on their amino acid sequences, amino acid composition and molecular weight, gliadin components can be classified in three groups: ω-, α / β- and γ-gliadins. Initially the α- and β-gliadins were classified in the same group on the basis of mobility at low pH in gel electrophoresis (Wieser 1996). Later studies have shown that the electrophoretic mobility does not always reflect the protein relationships and as a matter of fact α- and β-gliadins fall into one group. The separation of gliadin fraction into more than hundred components is possible due to methods such as two-dimensional electrophoresis and reversed-phase high-performance liquid chromatography (RP-HPLC). Based on the analysis of complete or partial amino acid sequences, amino acid compositions and molecular weights, gliadins can be grouped into four different types: ω5-, ω1,2-, α / β- and γ-gliadins.

Table 2. Characterization of gluten protein types. Adapted from Wieser (2007).

Type	**MWx10^{-3}**	**Proportions [1] %**	**Partial amino acid composition %**				
			Gln	**Pro**	**Phe**	**Tyr**	**Gly**
ω5-gliadins	49-55	3-6	56	20	9	1	1
ω1,2-gliadins	39-44	4-7	44	26	8	1	1
α / β-gliadins	28-35	28-33	37	16	4	3	2
γ-gliadins	31-35	23-31	35	17	5	1	3
x-HMW-GS	83-88	4-9	37	13	0	6	19
y-HMW-GS	67-74	3-4	36	11	0	5	18
LMW-GS	32-39	19-25	38	13	4	1	3

[1]According to total gluten proteins

GS = glutenin subunit

Types ω5- and ω1,2-, as well as α/β- ja γ-gliadins are related (Shewry and Tatham 1999). Within each type, structural differences are small and concern only substitution, deletion and insertion of single amino acid residues (Wieser, 1996). ω-gliadins contain mostly glutamine, proline and phenylalanine, which together account for for around 80 % of the total composition (Shewry et al. 1986; Wieser 1996). ω-gliadins are stabilized by strong hydrophobic interactions (Tatham and

Shewry 1985). In comparison to ω1,2-gliadins, ω5-gliadins have bigger molecular weight (~50000) and higher quantity of glutamine and phenylalanine (Wieser 1996). Most ω-gliadins lack cysteine, thus there is no possibility to form disulphide crosslinks. They consist almost entirely of repetitive sequences that are rich in glutamine and proline, for example PQQPFPQQ. ω-gliadins are rich in β-turns, with no detectable α-helix or β-sheet (Tatham and Shewry 1985).

Molecular weights of α/β- and γ-gliadins are between 28000 and 35000 (Table 2). Proportions of glutamine and proline are much lower in comparison to ω-gliadins. They differ significantly in the contents of some amino acids, such as tyrosine. Whilst the ω-gliadins are stabilized by strong hydrophobic interactions, the main stabilizing forces for the α/β- and γ-gliadins are covalent disulphide bonds and non-covalent hydrogen bonds (Tatham and Shewry 1985).

The repetitive units of α/β-gliadins are dodecapeptides such as QPQPFPPQQPYP and they are usually repeated five times and modified by the substitution of single residues (Wieser 1996). For γ-gliadins, the typical unit is QPQQPFP. It is repeated up to 16 times and some additional residues can be found within. The N-terminal domain is 40-50% of total proteins and consists mostly of repetitive sequences rich in glutamine, proline, phenylalanine and tyrosine and is unique for each type (sequence sections I and II, Figure 1). The N-terminal domains of α/β- and γ-gliadins are characterized by β-turn conformation, that is similar to ω-gliadins (Tatham and Shewry 1985). Within the C-terminal domains (sequence sections III-V, Figure 1), α/β- and γ-gliadins are homologous. With some exceptions, α/β-gliadins contain six cysteines in the C-terminal domain, while γ-gliadins contain eight of those (Grosch and Wieser1999). The non-repetitive C-terminal domain contains considerable proportions of α-helix and β-sheet structures and have less glutamine and proline in comparison to the N-terminal domain (Tatham and Shewry 1985). The helices have high stability, which is probably due to extensive hydrogen bonding, although disulphide bonds are also important.

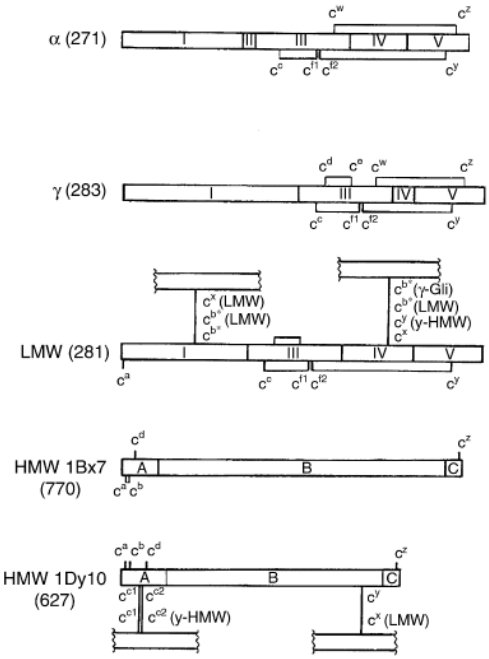


Figure 1. Disulphide structures of α/β- and γ-gliadins, and LMW- and HMW-glutenin subunits (x and y). Adapted from Grosch and Wieser (1999).

Though the distribution of total gliadins among the different types is dependent on wheat variety and growing conditions (soil, climate and fertilization), it can be generalized that α/β- and γ-gliadins are more abundant and the ω-gliadins occur in much lower proportions (Wieser and Kieffer 2001).

A portion of gliadins have an odd number of cysteines due to mutations and are linked together or to glutenins (Huebner and Bietz 1993). They appear either in alcohol-soluble oligomers in the gliadin fraction or in the alcohol-soluble glutenin polymers. This oligomeric fraction has been called HMW-gliadin, aggregated gliadin or ethanol-soluble glutenin (ESG). It contains α/β- and γ-gliadins, and LMW-subunits linked by interchain disulphide bonds. Their molecular weight is around 100000-500000. After reduction, ESG subunits elute during RP-HPLC in the same area as monomeric gliadins.

2.2.3 Glutenins

Before, only gliadin was classified as wheat prolamin (Shewry and Tatham 1999). According to the Osborne protein solubility classification, prolamins are a fraction of gluten, which are soluble in aqueous ethanol and insoluble, or nearly insoluble in water. They are readily soluble in acids or alkalis. Now it is accepted that the major wheat proteins defined as glutenins by Osborne, are in fact prolamins too, although they are insoluble in ethanol in their natural state. This is due to the fact,

that they are present in high molecular mass polymers stabilized by inter-chain disulphide bonds in addition to the intrachain bonds (Shewry 2003; Shewry and Tatham 1999). Once these bonds are reduced the subunits become soluble in ethanol.

Glutenins are present in the grain as aggregates stabilised by covalent disulphide bonds (Shewry et al. 1986). When the disulphide bonds of glutenins are reduced, the resulting glutenin subunits show a solubility in aqueous alcohols similar to gliadins (Wieser and Kieffer 2001). Likewise to gliadins, glutenins contain high proportions of proline and glutamine (Shewry et al. 1986; Shewry and Tatham 1999). Glutenins can be classified into two groups: the high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). LMW-glutenin is the predominant protein type of gluten and their proportion is ~20% of total gluten proteins, whereas HMW glutenin subunits are minor components within the gluten protein family (~10%) (Wieser and Kieffer 2001; Wieser 2007). LMW-glutenins are similar to α/β-gliadins and γ-gliadins for their molecular weight (MW) and amino acid composition (Table 2). HMW-glutenins can be classified in x- and y-types based on their molecular weight (Table 2). X-HMW-glutenins have a molecular weight of 83000-88000, whereas for y-HMW-glutenins it is between 67000 and 74000 (Wieser 1996). Dough properties are strongly affected by the quantities of HMW-GS and the contribution of the x-type to dough properties has been found to be more important than the y-type (Wieser and Kieffer 2001).

Similar to α/β- and γ-gliadins, LMW-GS contain two different domains: The N-terminal domain consists of glutamine- and proline-rich repetitive units, such as QQQPPFS (sequence section I, Figure 1). The other, C-terminal domain is homologous to that of α/β- and γ-gliadins within sections III and V (Figure 1). LMW-GS contain eight cysteines (Grosch and Wieser 1999). Six residues are in positions homologous to α/β- and γ-gliadins, thus they are proposed to be linked by intrachain disulphide bonds. Two additional cysteine residues that are unique to LMW-GS are located in sections I and IV (Figure 1). They are not able to form intrachain bonds, whereas interchain disulphide bonds with cysteines of different gluten proteins are generated.

HMW-GS consist of three structural domains (Figure 1): a non-repetitive N-terminal domain (A) comprising about 80-105 residues, a repetitive central domain (B) comprising about 480-700 residues and a non-repetitive C-terminal domain (C) comprising 42 residues (Shewry et al. 1992). Domain B contains repetitive hexapeptides (QQPGQG) as a backbone with some inserted

hexapeptides, such as YYPTSP, and tripeptides, such as QQP or QPG. Studies indicate the presence of β-reverse turns within the domain B (Shewry et al. 1992). These were predicted to be overlapping and form a loose spiral which was assumed to contribute to the elasticity of gluten. The domains A and C are proposed to have globular structures containing α-helices.

The domains A and C have most or all of cysteines: The x-type HMW-GS (except the subunit Dx5) has three cysteines in domain A and one in domain C and the y-type has five cysteines in domain A and one cysteine in each of domains B and C (Shewry and Tatham 1997; Shewry et al. 1992). The adjacent cysteines of domain A are connected to the corresponding residue of another y-type with interchain bonds and the cysteine of domain B is linked to a cysteine of LMW-GS. Two residues of domain A are linked by an intrachain and the other two by interchain disulphide bonds (Shewry et al. 1992).

2.2.4 Wheat prolamin antibody epitopes

Antibodies are directed against epitopes that are specific determinants of antigens (Osman et al. 2001). Knowledge of the epitope recognized by antibodies is an important basis for their proper use in immunological assays. The reactivity of antibodies R5 and G12 was investigated against gluten protein types. Also the reactivity of antibody Skerritt was investigated, but none of its epitopes were found from the gluten proteins. Based on this table, ω-gliadin had the most of the reactive epitopes (Table 3). Glutenins did not include the epitopes presented in here.

Table 3. Reactivity of R5 and G12 antibodies to gluten protein types. Uniprot accession is below each protein.

Antibody	Epitope	HMW-GS (B1B520)	LMW-GS (B2Y2R6)	ω-gliadin (A0A060N0S6)	α-gliadin (K7WV47)	γ-gliadin (R9XUS6)
R5	QQPFP	-	-	15	1	5
	LQPFP	-	-	-	-	-
	QLPYP	-	-	-	2	-
	QLPTF	-	-	-	-	-
	QQSFP	-	-	2	-	-
	QQTFP	-	-	-	-	1
	PQPFP	-	-	-	1	-
	QQPYP	-	-	1	1	-
G12	QPQLPY	-	-	-	1	-
	QPQLPF	-	-	-	-	-
	QPQLPL	-	-	-	-	-
	QPQQPY	-	-	1	-	-
	QPQQPF	-	-	10	-	6

2.3 Gluten detection in gluten-free foods

The gluten quantification method in foods and ingredients shall be based on an immunologic method or other method providing at least equal sensitivity and specificity (CAC 2008). The antibody used in the method should react with the specific cereal protein fractions that are toxic for persons intolerant to gluten and they should not cross-react with other cereal proteins or other constituents of the foods or ingredients. Methods used should be validated and calibrated against a certified reference material and the detection limit of the method should be 10 mg gluten/kg or below. Codex Alimentarius Commission recommends enzyme-linked immunoassay (ELISA) R5 Mendez method for gluten quantification in foods and ingredients.

2.3.1 Extraction

In cereal science the sample is usually wheat flour, dough or a finished product, and in almost all cases the sample has to be dried and ground before extraction (Hamer 2003). Drying is a necessary step to ensure the stability of the protein during storage, but it should not influence the protein.

Another issue is the particle size of the sample to be extracted, since particle size is related to the surface area and hence can affect the extraction. As a rule of thumb, the sample should be milled to pass a 0.5 mm sieve. Finally, a point of concern is related to the lipid content of the sample. For example, the high fat content of oats can cause problems in many wet fractionation procedures. For this reason, the samples are often defatted before the extraction procedure.

The need for improved sample extraction methods has led to the development of new extraction solutions containing reducing agents, such as 2-mercaptoethanol, which in combination with alcohol extraction are capable of dissolving gluten aggregates (Valdés et al 2003; Mena et al 2012; Kanerva et al 2011). The cocktail solution is recommended as the extraction solution for heated foods and it is a part of the R5-ELISA kit (García et al. 2005). After the extraction procedure with the cocktail solution the sample prolamins can still react with the antibody (Valdés et al. 2003).

2.3.2 The sandwich and competitive ELISA

The determination of prolamins by enzyme-linked immunosorbent assay (ELISA) and the conversion of the resulting concentration to gluten content in food is a method used by many laboratories (Diaz-Amigo and Popping 2013). There are two ELISA formats, sandwich and competitive. The principles of the different formats are presented in figure 2. The sandwich method is based on two antibodies (Valdés et al. 2003). The first one (capture antibody) is bound to the bottoms of the microplate wells, in known quantity. Then the antigen-containing sample is added and antigen/antibody complex is formed (step 1). Next, excess antigen is removed by washing. The second antibody labelled with an indicator enzyme (detecting antibody) is added and it detects the antigens attached to the capture antibody. For example horse radish peroxidase or alkaline phosphatase are commonly used as indicator enzymes. The antigen is “sandwiched” between the capture antibody and the detecting antibody (step 2). The unbound detecting antibodies are washed out and enzymatic substrate is added (step 3). The enzyme induces a colour reaction, which can be measured by spectrophotometric methods. The sample protein must be large enough for two antibodies to attach to it at the same time and this is why sandwich ELISA cannot be used for hydrolysed gluten samples.

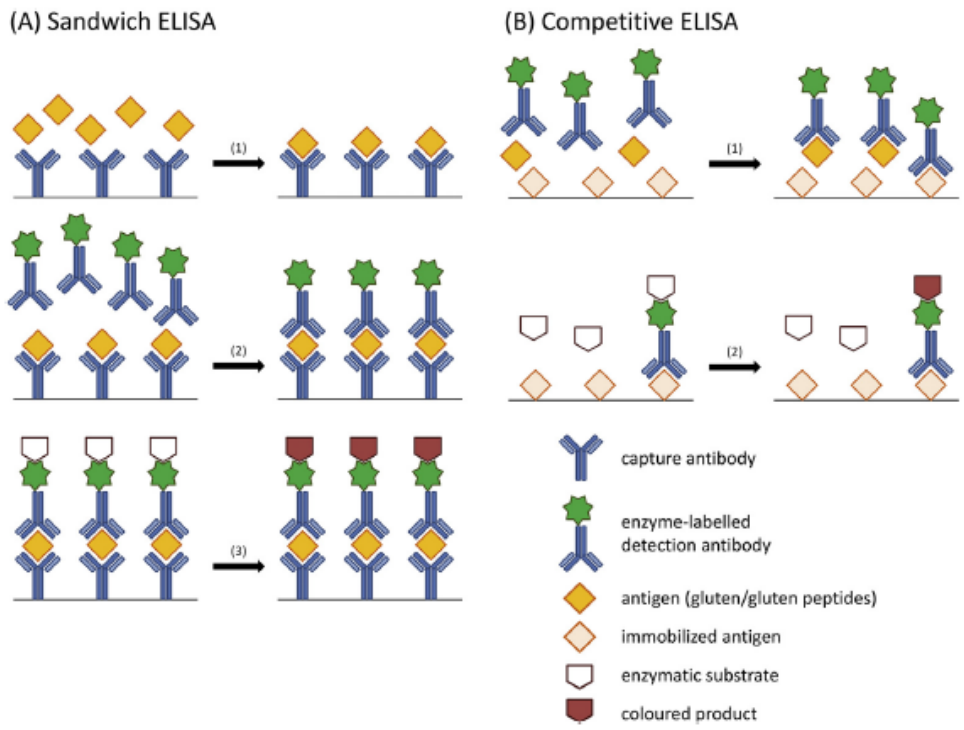


Figure 2. The principles of (a) sandwich ELISA and (b) competitive ELISA. Adapted from Scherf and Poms (2016).

A commercial competitive ELISA R5 was developed to determine gluten content in hydrolysed products, such as beer (Bermudo Redondo et al. 2005). The peptide profile of hydrolysed gluten proteins depends on the type of hydrolysis, extent of hydrolysis and enzyme cleavage points (Diaz-Amigo and Popping 2013). The difficulties of converting hydrolysed peptide concentration in true gluten content make the quantification of gluten challenging. Competitive ELISA is more suitable for the detection of hydrolysed gluten because it requires only one antibody binding epitope instead of two (Bermudo Redondo et al. 2005). This way smaller peptide fragments can be detected.

In the competitive ELISA only one antibody is used and the method is based on the competition between sample proteins and standard proteins (Haas-Lauterbach et al. 2012). In figure 2b is shown the method. Known quantity of antigen is applied on the surface of the microtiter plate. The antigen-containing sample and an enzyme-labelled antibody are applied at the same time to the well. During incubation, the immobilized and free antigens compete for the binding sites (step 1). After washing, enzymatic substrate is added to form a coloured product (step 2). Because of only one antibody, competitive ELISA is also suitable for detecting small, hydrolysed proteins and peptides. However, the specificity is not as good in comparison to the sandwich method, since nonspecific binding is more likely when only one binding site is needed for detection. In the competitive method, the enzyme can be conjugated with the antibody or with the standard protein.

In both of the ELISAs, a calibration curve with gluten peptide is used to calculate the antigen concentration in the sample extract. To comply with the units established with Codex Standard 118, analytical methods specific to prolamins require a conversion factor of two to convert prolamin content into gluten concentration (Diaz-Amigo and Popping 2013). The factor is set to two, because gliadin is considered to be half of the gluten proteins the remaining half being the glutenin fraction.

2.3.3 Antibodies

The currently most widely accepted method for gluten quantification is ELISA based on the R5 antibody (CAC 2008). Among the antibodies from commercial kits, R5 reacts stronger against gliadins (Sharma 2012). R5 is a monoclonal antibody that has been raised against rye extracts (Sorell et al. 1998). ELISA R5 was presented as a new option for gluten quantification in the late 1990s (Diaz-Amigo and Popping 2013). R5 specifically detects as core epitope the sequence that can be found in gliadin peptides with known toxicity for people suffering from gluten intolerance: QQPFP, QQQFP, LQPFP and QLPFP are the strongest target epitopes and they are also present in α/β-, and γ-gliadins (Kahlenberg et al. 2006). Some of the R5 epitopes can be also found from LMW glutenins, which are related to α/β- and γ-gliadins in molecular weight and in amino acid composition (Wieser 2007).

G12 is a monoclonal antibody that has been raised against the synthetic 33-mer toxic α-gliadin peptide, QPQLPY being the most reactive epitope (Morón et al. 2008). This antibody binds prolamins from wheat, rye, barley and some oat cultivars. With G12, there is an opportunity to also protect the small percentage of the population sensitive to oats. The Skerritt antibody is the oldest of the antibodies mentioned (Diaz-Amigo and Popping 2013). It was used in some commercial ELISAs until some disadvantages became known and new developments led to the use of other antibodies and analytical approaches. Skerritt has been raised against ω-gliadin, but it also shows high reactivity with the glutenin fraction (Sharma 2012). The most recently developed monoclonal antibody is α20, which was generated against the coeliac disease-immunogenic peptide PFRPQQPYPQP from α-gliadins (Mitea et al. 2008). Gliadins, secalins and hordeins are recognized by the α20, but there is limited evidence on other reactivities. There are also ELISA kits that use polyclonal antibodies (Morinaga) (Diaz-Amigo and Popping 2013). Antibodies in this kit are raised

against a commercial gliadin preparation. There is not much information about this kit in the scientific literature, but the assay is mostly specific to gliadins (Sharma 2012).

2.3.4 PWG gliadin as reference material

At the moment the most recommended reference material for prolamin quantification is gliadin from Prolamin Working Group (PWG) (Lexhaller et al. 2016). It is a selection of 28 European wheat cultivars, with 86,4 % gliadin content, including 41,7 % α-gliadin, 47 % γ-gliadin and 11,3 % ω-gliadin (Figure 3). The cultivars were selected from the three main European wheat-producing countries, France, UK and Germany. One kilogram of kernels from each cultivar were mixed, milled and the resulting white flour was defatted and vacuum dried. Albumins and globulins were eliminated by extraction using NaCl solution. Gliadins were extracted with 60 % ethanol. The resulting gliadin extracts were concentrated, desalted by ultrafiltration, freeze-dried and homogenized. The reference gliadin was sent to 16 different laboratories for further investigations. The results showed that the gliadin composition of the source flour and the reference gliadin matched perfectly, thus no major gliadin components had been lost during the isolation procedure. The results also showed that the reference gliadin had good immunochemical sensitivity with different gliadin antibodies in enzyme immunoassays. Since it has high protein and gliadin content, good solubility, homogeneity, stability and representative character, the gliadin reference material is regarded as a suitable universal reference material.

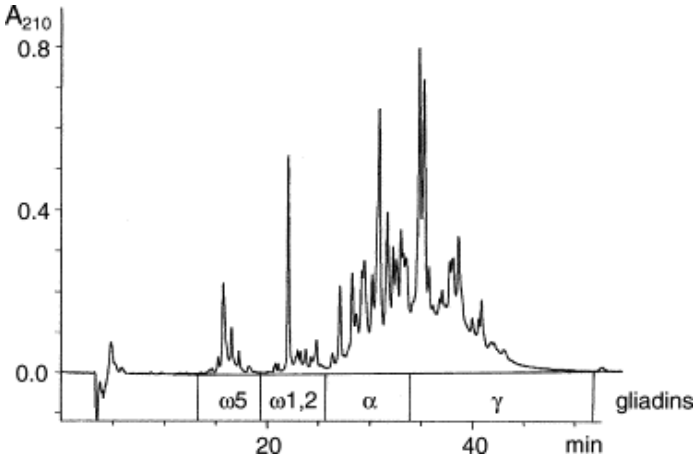


Figure 3. RP-HPLC of the PWG-gliadin (250 µg/100 µl) on C_8-silica gel, peaks for the gliadin types are indicated. Adapted from van Eckert et al (2006).

2.4 The challenges in gluten detection

In the absence of other solutions, the ELISA R5 and the PWG gliadin were adopted as reference method and gliadin material for gluten quantification with no opposition (Diaz-Amigo and Popping 2013). The performance of new ELISA developments is compared to the R5 method. It should be taken into consideration that an assay validated in multiple multilaboratory studies and always under the same conditions does not guarantee a good real-life performance, where analytical conditions, such as food matrices and gluten sources, are variable. The measuring of gluten content is particularly challenging when the source of gluten is unknown (Lexhaller et al. 2016).

2.4.1 Assay target and antibody specificity

In a study by Lexhaller et al. (2016) the specificity and sensitivity of three monoclonal (R5, G12 and Skerritt) and two polyclonal antibodies against prolamin and glutelin fractions from wheat, rye and barley were compared. Reversed-phase high-performance liquid chromatography (RP-HPLC) was used as an independent reference method for the quantification of protein contents in the gluten fractions. The estimated prolamin, glutelin and gluten contents quantified by RP-HPLC versus those by five different ELISA kits are listed in table 4. Calculation factor of 2 is used for R5 and pAb1 in order to obtain the whole gluten content, since these antibodies are believed to recognize only gliadins (Lexhaller et al. 2016). For pAb2, calculation factor of 0.85 is recommended since it uses wheat proteins for calibration and with the calculation factor, gluten content is obtained. The results show that there is variation in the estimation of the prolamin content between the methods.

Table 4. Estimated prolamin, glutelin and gluten contents quantified by RP-HPLC versus those by ELISA. PWG-gliadin was used as a reference material. Adapted and modified from Lexhaller et al. (2016).

Method	Prolamin content (mg / kg)	Glutelin content (mg / kg)	Gluten content as sum of prolamin and glutelin contents (mg / kg)	Gluten content as sum considering the recommended calculation factors (mg / kg)
RP-HPLC	10	10	20	20[a)] / 20[b)] / 17[c)]
R5	14	1	15	28[a)]
G12	16	2	18	18[b)]
Skerritt	13	93	106	106[b)]
pAb1	11	2	13	22[a)]
pAb2	21	13	34	29[c)]

[a)] Calculation factor for R5 and pAb1 = 2 x prolamin content

[b)] No additional calculation factor

[c)] Calculation factor for pAb2 = 0.85 x sum

The specificity of monoclonal antibodies R5, Skerritt, G12 and α-20 is evaluated mainly against wheat gliadins, although gliadin is not the only target of antibodies currently used by some assays. (Diaz-Amigo and Popping 2013). This induces a high degree of uncertainty and potential assay inaccuracy. It also has to be taken into account that when the results of newly developed assays differ from the results of ELISA R5, it may not necessarily indicate that they are unreliable. With all the unknowns linked to ELISA methods, it is difficult to say which assay is more accurate. There are also unanswered questions of what is the fragment size recognized by the antibodies that still triggers an effect in coeliac patients (Diaz-Amigo and Popping 2013). Codex Standard states "the antibody used should react with the cereal protein fractions that are toxic for persons intolerant to gluten". It is also possible that antibodies in competitive assays also recognize fragments that do not trigger coeliac disease but still contribute to the ELISA signal, preventing the labeling of some products as gluten-free.

2.4.2 Reference material

Wheat as reference material is not necessarily suitable to determine gluten from rye or barley. There is evidence that for example for barley, R5 antibody cannot provide accurate results (Huang et al. 2017, Kanerva et al. 2006, Tanner et al. 2013). In a study by Huang et al. (2017) the prolamin

concentrations of gluten-free oat flour spiked with barley flour were determined by HPLC, ELISA R5 with gliadin standard and ELISA R5 sandwich with 40% C-hordein standard (Figure 4). The concentration of barley prolamin, hordein, in gluten-free products was overestimated 1.8-2.5 times by the R5 ELISA method when calibrated against the wheat gliadin standard. Estimates in the correct range were obtained when the standard was 40% C-hordein. One reason for the overestimation was assumed to be the different composition of gliadin reference material from that of hordeins.

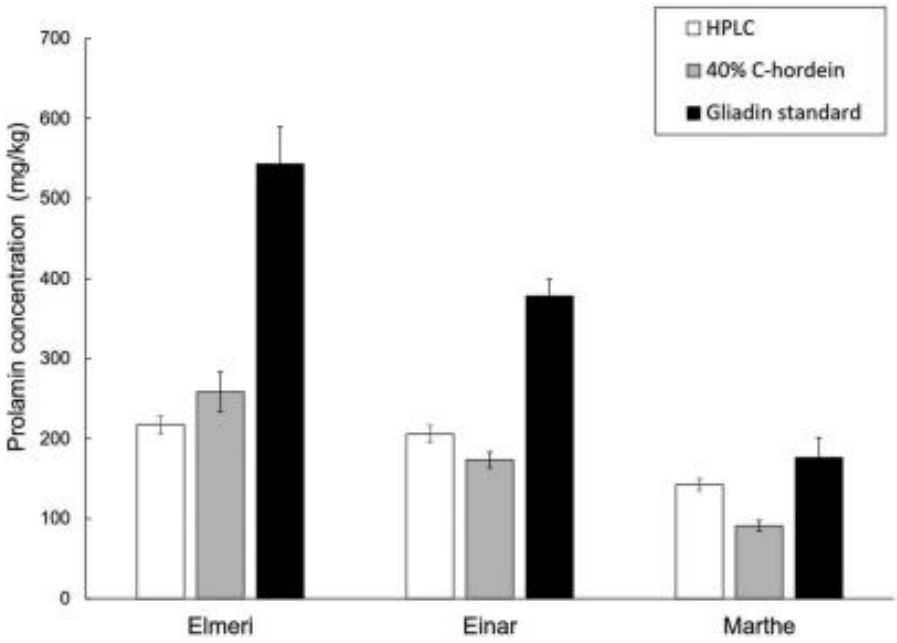


Figure 4. Prolamin concentration of gluten-free oat flour spiked with three barley flours, determined by HPLC, R5 ELISA sandwich with 40% C-hordein standard, and R5 sandwich ELISA with gliadin standard. Adapted from Huang et al. (2017).

In this study, the recognition of whole hordein by R5 antibody mainly came from C-hordein, which is more reactive than the other hordeins. The proportion of C-hordein in total hordein ranged from 16 to 33% of common Finnish barley cultivars and was always higher than that of ω-gliadin, the homologous protein class in the gliadin standard, which may account for the overestimation. The reactivity of D-, C-, and B-hordeins against R5 antibody varied widely in ELISA (Figure 5). C-hordein was 10-20 times more reactive than gliadin standard, which was 8-25 more reactive than B-hordein. The curves indicated that C-hordein and gliadin standard had similar affinity with the R5 antibody.

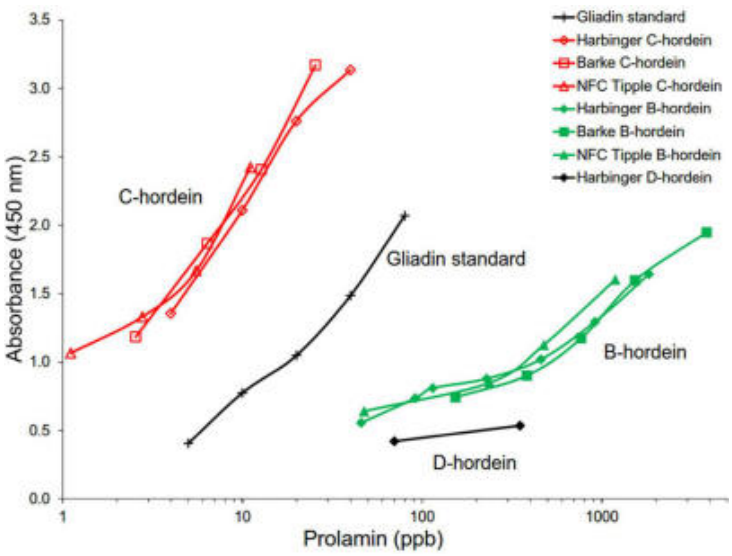


Figure 5. Reaction of isolated hordeins against R5 antibody in sandwich ELISA (Huang et al. 2017).

ELISA R5 has been calibrated and validated based on the PWG gliadin and hence it is expected to provide accurate results (Diaz-Amigo and Popping 2013). However, it cannot provide accurate results for sample materials that have different prolamin compositions. Another issue with the PWG gliadin reference material is that it is difficult to reproduce because the composition of gluten varies from season to season because of the differences in soil and weather conditions.

Therefore, what would be the most suitable reference material for gluten detection? It is a challenging task for several reasons: (1) gluten consists of several large proteins that can be potential targets for detection methods; (2) these proteins have physicochemical differences; (3) the expression of these proteins depends on cereal cultivar and growing conditions; (4) the structure and solubility of the gluten-containing products are modified during food manufacturing (Wieser 2007; Diaz-Amigo and Popping 2013). Reference material should contain all of the assay targets and it should be commutable, that is to say, it should cover the needs of the several existing detection methodologies. (Diaz-Amigo and Popping 2013). One solution would be to have one reference material per grain, which could be used to evaluate assay response to the individual grains and also allow making of customized mixtures. Or another approach would be to select and characterize a gluten material, that is, gluten or flour with characterized gluten content. On the other hand, using a material having only a single prolamin fraction would be useful only to the assays specific for those fractions, and another reference material should be developed for other assays. Nonetheless, reference material should be as close to real product as possible.

To help deal with the variety of the ELISA kits and to ensure the accuracy of the analytical results, a new gluten reference material is needed. Historically the gliadin fraction has been reported to be the unique gluten fraction responsible for the toxicity of cereals in coeliac patients and very little attention has been paid to glutenin fraction or gluten as whole (Howdle 2006). As a result, the search for reference materials has been limited to gliadin, with very little consideration to the use of gluten or other cereal sources such as rye or barley.

2.4.3 Conversion factor of 2

To ensure that the analytical tools are used properly, it is necessary to ensure that the interpretation of regulations is accurate. For example, there is a frequent misinterpretation considering the units of the action level, which is set by the Codex Standard and the EU Regulation: the labeling threshold is 20 mg of gluten/kg, not 10 mg of prolamin/kg, which is frequently and wrongly set as equal (Diaz-Amigo and Popping 2013). This misinterpretation occurs because most ELISAs measure prolamin, which is then converted into gluten by simply multiplying the prolamin content by a factor of two. Converting prolamin into gluten is not straightforward and can lead to inaccuracy of the results.

The ratio of prolamin to glutelin varies among different cereals and within a cereal depending on the cultivar and growing conditions (Wieser and Koehler 2009; Diaz-Amigo and Popping 2013). In a study by Wieser and Koehler (2009) the ratio of prolamin to glutenins ranged from 1.32 to 1.66 for wheat, from 1.12 to 1.16 for rye and from 1.20 to 1.71 for barley. Therefore the direct implication of using a factor of two is an overestimation of the gluten content in food samples (Diaz-Amigo and Popping 2013). Also, the factor is only applicable when the assay is specific exclusively to gliadins and results are overestimated in assays that also detect glutenins.

In addition, there is only limited information about whether the toxicity of rye and barley is equivalent to that of wheat one to one (Diaz-Amigo and Popping 2013). Action levels for gluten-free (20 ppm) are based on the toxicity of wheat and there is a nonscientific assumption that the same concentrations of rye and barley are more, less, or equally safe to coeliac patients compared to wheat.

3 AIMS OF THE STUDY

The aim of this study was to compare the prolamin compositions of different wheat cultivars and their reactivity against R5 antibody in sandwich ELISA. The aim was to isolate total gluten and to find the most suitable ratio of C-hordein to be used as a reference material for wheat gluten quantification of the selected cultivars instead of current gliadin standard. The hypothesis was that there would be great variation in the prolamin composition between different wheat cultivars and that the current gliadin reference material would not be suitable to represent all of them. The specific aims of the study were to:

- Optimize the method for the extraction of gliadin and glutenin and separation by RP-HPLC
- Categorize the wheat cultivars by their gliadin peak patterns and calculate their ω-gliadin proportions by integrating the peak areas
- Do separation and collection of prolamin groups by RP-HPLC and analyse the fractions by SDS-PAGE
- Isolate total gluten of the wheat cultivars and analyse their reactivity against R5 antibody by sandwich ELISA R5
- Try to find optimal percentage of C-hordein to be used as a reference material

4 MATERIALS AND METHODS

4.1 Materials

Twenty-three wheat cultivars were selected for this study. The cultivars were selected to be used in the study, since they were the most prevalent wheats of each country. Cultivar Julius was kindly provided by KWS (Germany) and cultivars Patras and Kerubino by IG Pflanzenzucht (Germany). Cultivars Siskin and Lili were from KWS (UK). Britannia, Claire, Zulu, Revelation and Crusoe were provided by Limagrain (UK). Cultivars Suntop, Spitfire, Lancer, Gregory and Mace were from Plant Breeding Institute, University of Sydney (Australia). Cultivars Julius, Brons and Hereford were kindly provided by Lantmännen (Sweden), Quarna and Anniina by Boreal Plant Breeding Ltd (Finland) and Amaretto by Planatanova (Finland). Apache and Cellule were provided by Maison Florimond Desprez S.A.S. (France). In addition, cultivar Annina (Finland, 26.1.2015) was used in some preliminary tests. The wheat kernels were milled to flour with a KT-3600 disc mill (Koneteollisuus Oy, Finland).

Extracted barley C-hordein with BSA (10, 20 and 30 % of C-hordein) was kindly provided by Xin Huang. All of the chemicals used were of analytical quality.

4.2 Workflow

The workflow of the study is presented in figure 6.

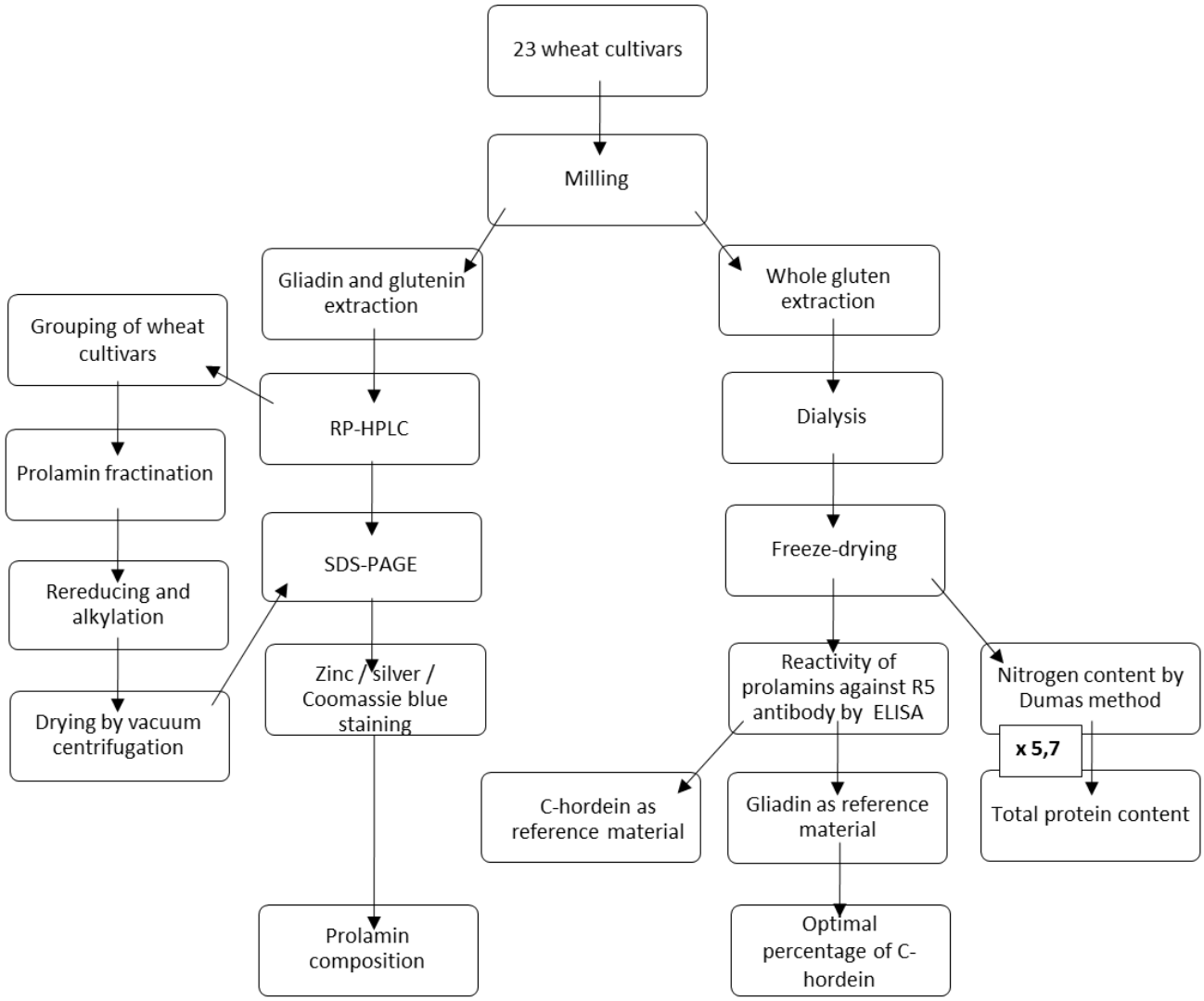


Figure 6. Workflow of the study.

4.3 Extraction

4.3.1 Gliadin and glutenin

Wheat flour (0,1 g) was extracted following the Osborne procedure of a) salt solution (2 x 1.0 ml: 0.4 M NaCl with 0.067 M $HKNaPO_4$, pH 7.4) for 10 min at ambient temperature to remove the albumin and globulin fractions; and b) with 1-propanol/water (2 x 0.5 ml: 50/50 v/v, for 10 min at 60°C to extract gliadins. The pellet was washed with MilliQ-water and centrifuged (5810R, Eppendorf AG., Germany) for 5 min at 20000 g to remove any buffer residues. Finally, c) glutelin solution (1 ml:1-propanol (50/50 v/v)/ 0.05 M Tris-HCl, pH 7.5, containing 2 M (w/v) urea and 1% (w/v) DTT for 20 min at 60°C was used to extract the glutenin fraction. Each extraction step was initiated with vortexing at room temperature. The suspensions were centrifuged for 10 min at 20000 g and the corresponding supernatants were combined. Aliquots of the extracts were filtered through a 0,45µm membrane before HPLC analysis.

4.3.2 Whole gluten extraction and dialysis

Whole gluten of all the milled wheat cultivars was extracted. Wheat flour (3 g) was mixed with 30 ml of 0.4 M NaCl with 0.067 M $HKNaPO_4$ (pH 7.4) and incubated for 15 min at room temperature in shaker. The samples were centrifuged for 30 min 18000 g at 20°C. The samples were washed with milli-Q water and centrifuged again. 30 ml of 1-propanol and water (50/50 v/v) with 0.2 % DTT (w/v) was added and the samples were incubated for 45 min at 60°C. The samples were centrifuged for 30 min at 18000 g at 20°C.

Large container was filled with milli-Q water. The samples were poured to dampen dialysis tubing cellulose membranes (SnakeSkin Dialysis Tubing, 7K MWCO, 22 mm, Thermo Fisher Scientific, USA). For the second set of gluten samples dialysis another dialysis membrane, Cellu-Sep T1/Nominal MWCO 3500 (Membrane Filtration Products, Inc., Texas) was used. The membranes were closed with clips and laid in the container. Water was changed after 5 hours and few drops of acetic acid was added with magnetic stirrers. The containers were covered with plastic wrap and left in the cold room for 2 days. The water was changed for two times again and 2 ml of acetic acid was added each time.

The samples were placed to plastic containers and first left to -20°C freezer for 2 h. Next they were left to -80°C freezer for 3 days. Finally the samples were placed in the freeze-dryer (Dura-Dry, FTS Systems, Inc.) for 4 days.

4.4 Total protein content

The nitrogen content of the extracted and freeze-dried wheat gluten samples of different cultivars was analysed by the Dumas method, which was based on the detection of total nitrogen in the sample. Due to the small sample size of some cultivars, 60-120 mg of gluten sample was weighted and the total protein content was analysed without replicates by Vario MAX CN Element Analyzer (Elementar Analysensysteme GmbH, Germany). Therefore statistical analysis could not be conducted. The nitrogen content was multiplied by the factor of 5.7 to calculate the crude protein content. The protein content was taken into account with the calculations of ELISA prolamin content measurements.

4.5 RP-HPLC method establishment

All the samples were analysed with Agilent Technologies Inc. 1200 series RP-HPLC (Germany) using a C8 column (4.6 mm X 250 mm, 300 Å, 5µm, Discovery, Sigma-Aldrich Co. LLC). Following preliminary tests were conducted:

- **Solvent.** The extraction of gliadin and glutenin was performed with a) 1-propanol and water (2 x 0.5 ml: 50/50 v/v); b) 2-propanol and water (2 x 0.5 ml: 50/50 v/v); and c) ethanol and water (2 x 0.5 ml: 60/40 v/v) to investigate the best extraction result. For glutenin extraction, also 2-mercaptoethanol (5% v/v) was used. An injection volume of 10 µl of both gliadin and glutenin extracts from wheat cultivar Julius (SWE) was separated on a RP-HPLC C8 column at 50°C.

- **Temperature.** Gliadin and glutenin extracts were analyzed in RP-HPLC at a) 25°C and b) 50°C to investigate the most suitable temperature for separation. Gliadin was extracted with 1-propanol and water (2 x 0.5 ml: 50/50 v/v) and a solution of 1-propanol (50/50 v/v)/ 0.05 M Tris-HCl, pH 7.5, containing 2 M (w/v) urea and 1% (w/v) dithiothreitol (DTT) was used

to extract the glutenin fraction. An injection volume of 10 µl of both gliadin and glutenin extracts from wheat cultivar Julius (SWE) was separated on C8 column.

- **Glutenin injection volumes.** Glutenin extract was analyzed with injection volumes of a) 25 µl and b) 50 µl to investigate if higher injection volume would result in a better separation. Glutenin was extracted with the same 1-propanol solution as previously. Glutenin extract from wheat cultivar Crusoe (UK) was separated on a C8 column at 50°C.

- **Gradient.** Gliadin extract was analyzed with three different gradients to investigate if a lower percentage of acetonitrile in the mobile phase would result in a better separation of ω-gliadin. The gradients are presented in table 5. Only cv. Crusoe was analyzed with the different gradients. Gliadin was extracted with 1-propanol and water (2 x 0.5 ml: 50/50 v/v). An injection volume of 25 µl of gliadin was separated on a C8 column at 50°C. Previously prepared samples were heated at 100°C for 1.5 min and vortexed.

Table 5. Preliminary tests. Different RP-HPLC solvent B% gradients for better gliadin separation of wheat cv. Crusoe. Solvent B = 0.1% TFA in acetonitrile. Solvent A = 0.1% TFA in milli-Q water.

Min	A	B	C
0	28	15	20
30	56	60	60
35[a)]	28	90	90
36	28	15	20

[a)] For gradient A 31 min.

4.6 Prolamin composition by RP-HPLC and SDS-PAGE

4.6.1 RP-HPLC of gliadin and glutenin extracts

Acetonitrile (ACN) and TFA were HPLC grade. An injection volume of 25 µl of the gliadin extract and 50 µl of the glutenin extract was separated on a C8 column at 50°C. A linear acetonitrile gradient shown in table 5 A was run over 30 min at a flow rate of 1 ml/min and the elution was monitored at 210 nm. The data was collected and the chromatograms were formed in Microsoft Excel. The cultivars were grouped based on similarities on their chromatograms. The gliadin peaks

were manually integrated and the peak areas were compared to determine the ω-gliadin proportion of total gliadin in each cultivar analysed by RP-HPLC.

4.6.2 SDS-PAGE

Gliadin and glutenin samples

Gliadin, glutenin and whole gluten extracts were analysed in SDS-PAGE. For gliadin and glutenin extracts, 40 µl of sample and 60 µl of SDS-buffer (62.5 mM Tris-HCl (pH 6.8) with 10% (v/v) glycerol, 2% (w/v) SDS, a hint of Bromophenol Blue) with 2-mercaptoethanol (5% v/v) was mixed in the Eppendorf-tubes. The tubes were vortexed and heated at 100°C for 3 min. 1-4 µl of the samples Crusoe, Revelation, Zulu, Claire, Britannia, Apache, Hereford, Brons and Julius (Sweden) and standard (Novex Sharp Pre-Stained Protein Standard, Thermo Fisher Scientific, CA) were loaded using a homogenous NuPAGE 10% polyacrylamide Bis-Tris gel (Invitrogen, CA, USA) and NuPAGE MOPS SDS Running Buffer (Invitrogen, CA, USA, pH 7.7). In addition, 8 µl of the samples Julius (Germany), Siskin, Lili, Suntop, Spitfire, Lancer, Gregory and Mace and standard were loaded using the Bolt 4-12% Bis-Tris Plus-gel (Invitrogen, CA, USA). The gels were attached to Mini Gel Tank (Thermo Fisher Scientific, Israel). The running conditions were 200 V for 50 min.

Gliadin with and without reducing agent

Another SDS-PAGE was conducted to gliadin extracts of cv. Julius (GER), Brons, Hereford, Apache, Britannia, Claire, Zulu, Revelation and Crusoe that had 1) 5% (v/v) mercaptoethanol added to the SDS-buffer and 2) no mercaptoethanol added. The ones without the mercaptoethanol were heated at 60°C for 10 min before adding the SDS-buffer. For each of the cultivars, 8 µl of sample was loaded and the running conditions were same as previously.

Whole gluten

To analyse the whole gluten samples in SDS-PAGE, 5 mg of sample was mixed with 500 µl of SDS-buffer with 5% (v/v) 2-mercaptoethanol and heated at 100°C for 3 min. The suitable amount of protein to be analyzed in SDS-PAGE was calculated and 3 µl of sample was decided to be loaded. SDS-PAGE was conducted as using the Bolt 4-12% Bis-Tris Plus-gels and SeeBlue Plus2 Pre-stained protein standard (Thermo Fisher Scientific Inc.) and XCell SureLock Mini-Cell

Electrophoresis System (Thermo Fisher Scientific Inc., China). The running conditions were 200 V for 40 min.

Coomassie Blue-staining

A staining solution was prepared (milli-Q water with 40% ethanol (v/v), 10% (v/v) acetic acid, 0,1% (v/v) Coomassie Brilliant Blue R-250) and added on the gels. They were heated in microwave for 1 min and then transferred to shaker for 15 min at room temperature. The gels were washed with milli-Q water. The gels were destained with 100 ml of the prepared de-staining solution (milli-Q water with 10% (v/v) ethanol and 7,5% (v/v) acetic acid) and heated in microwave for 1 min and left in mixer for 1-1,5 h at room temperature. The gels were washed with milli-Q water and analysed and photographed on a light table.

4.7 Characterization of wheat gluten protein types

4.7.1 RP-HPLC fractionation of gliadin and glutenin subgroups

The prolamin fractions of wheat cv. Crusoe were collected according to their characteristic retention times from the gliadin and glutenin extracts with a linear acetonitrile gradient shown in table 5 B (50°C). The collected fractions were dried first under a nitrogen stream at 40°C for 1 h and then by vacuum centrifugation (Savant SpeedVac SC110A concentrator, USA). The fractions were solubilized in 100 µl SDS-buffer with 5% (v/v) 2-mercaptoethanol and heated at 100°C for 3 min before SDS-PAGE. The load volume of each sample and standard (SeeBlue Plus 2 Pre-stained protein standard, Thermo Fisher Scientific, MA) was 3-12 µl and the same Bolt 4-12 % gel was used as previously. The run time was 50 min. Silver Stain Plus kit (Bio-Rad, Inc.) was used for the staining. The staining solution was let to affect for 19 min before removal.

Also other two sets of cv. Crusoe's gliadin fractions were collected, with a linear acetonitrile gradient shown in table 5 B and C. Lower percentage of acetonitrile in the solvent was assumed to result in a better separation since ω-gliadin is hydrophilic. The fractions were dried first under a nitrogen stream at 40°C for 1 h and then by vacuum centrifugation.

4.7.2 Rereducing, alkylation and SDS-PAGE of the collected prolamin fractions

Rereducing and alkylation was conducted to one set of collected and dried HPLC-fractions. DTT-solution (100 mM Tris-HCl (pH 8.3) with 5 mM DTT) was added to the tubes (25 µl), mixed and incubated for 15 min at 60°C. Iodoacetamide-solution (30 mM Tris-HCl with iodoacetamide) was added (25 µl) and incubated at room temperature for 30 min. Iodoacetamide is sensitive so all the tubes and solutions were covered in aluminum foils. Finally, SDS-buffer with 5 % 2-mercaptoethanol was added before SDS-PAGE.

The tubes were incubated at 60°C for 15 min. In addition to the gliadin fractions, an extracted whole gliadin- sample was analysed with SDS-PAGE and before loading, it was heated for 3 min at 100°C. The load volume of all the samples was 15 µl and run time 40 min. Silver staining was conducted as previously. The staining solution was let to affect for 21 min.

4.8 C-hordein as a reference material in sandwich ELISA

The wheat prolamins of different cultivars, C-hordein and gliadin standard were investigated against R5 antibody in sandwich ELISA (Ridascreen Gliadin, R-Biopharm AG, Germany). The previously extracted and freeze-dried wheat gluten samples of all the cultivars were weighted to Falcon tubes (10 mg) and 1.25 ml of cocktail solution (R-Biopharm AG, Germany) was added and vortexed. The samples were incubated at 50°C for 40 min in Incubator 1000 (Heidolph Instruments GmbH & CO, Germany) while mixing. The samples were cooled under running water and 3.75 ml of 80% (v/v) ethanol was added. The samples were vortexed and incubated for 60 min at room temperature in shaker (REAX 2000, Heidolph Instruments GmbH & CO, Germany). The samples were centrifuged for 10 min at 18000 g at 21°C.

The dilutions were done according to the manufacturer's instructions with the sample dilution buffer provided in the kit. The diluted samples, gliadin standards and barley C-hordein samples with BSA (10, 20 and 30% of C-hordein) were loaded (100 µl) to the microplate and incubated for 30 min. Plate washing was conducted with microplate washer for 3 times (Asys Hitech GmbH, Biochrom, ASYS Atlantis, Austria). Diluted enzyme conjugate was loaded (100 µl) to the wells and

incubated for 30 min at room temperature. The microplate was washed as previously. 50 µl of substrate and 50 µl of chromogen was added to each well, followed by incubation for 30 min at room temperature in the dark. Finally, 100 µl of the stop reagent was added to each well. The absorbance was measured by spectrophotometer (Labsystems, Original Multiscan EX, Finland) at 450 nm with Ascent Software Version 2.6 (Thermo Labsystems).

The response of the ELISA systems antibody to increasing prolamin concentration of the wheat gluten samples was plotted on a log axes. The crude protein content of the samples analysed by the Dumas method, was taken into account. The data was replotted as linear Michaelis-Menten plots and the Km values were determined from the curves of best fit (GraphPAD Prism 6). The Km values of prolamins were determined from the curves. The Km is a measure of the amount of prolamin required to produce a half-maximal response in the ELISA assay. The calculation was done by use of a cubic spline function. Averages were calculated with Microsoft Excel.

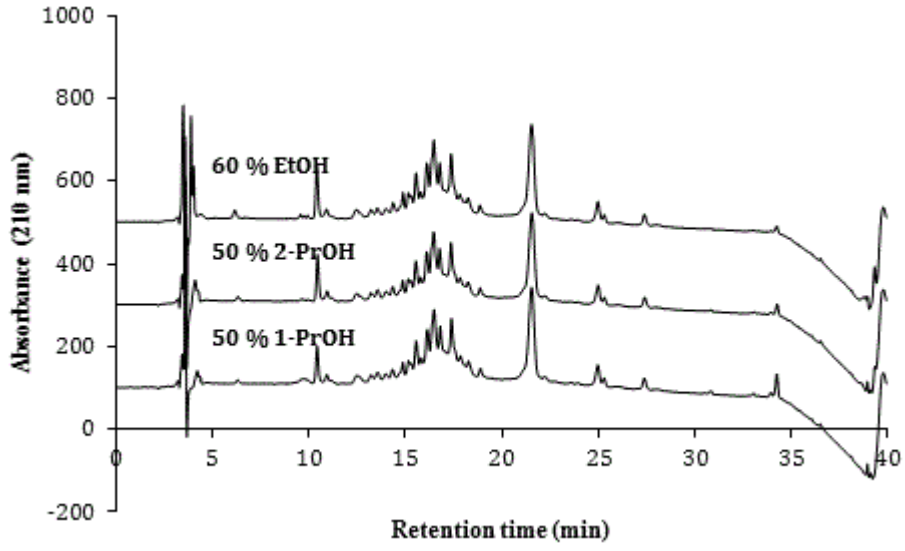
5 RESULTS

5.1 RP-HPLC method

5.1.1 Solvent

The extraction of gliadin and glutenin was performed with 1) 50 % 1-propanol (v/v), 2) 50 % 2-propanol (v/v) and 3) 60 % ethanol (v/v) to investigate which would result in best extraction. For glutenin extraction, also 5 % 2-mercaptoethanol (v/v) was used. The chromatograms of gliadin and glutenin extracts with different solvents used did not differ, thus the solvent had no effect on better gliadin or glutenin extraction (Figure 7). Gliadin peaks were well separated with all the solvents, whereas glutenin peaks were poorly separated and another solvent was decided to be tested. For gliadin extraction, 50 % 1-propanol (v/v) is to be used in the following experiments.

A



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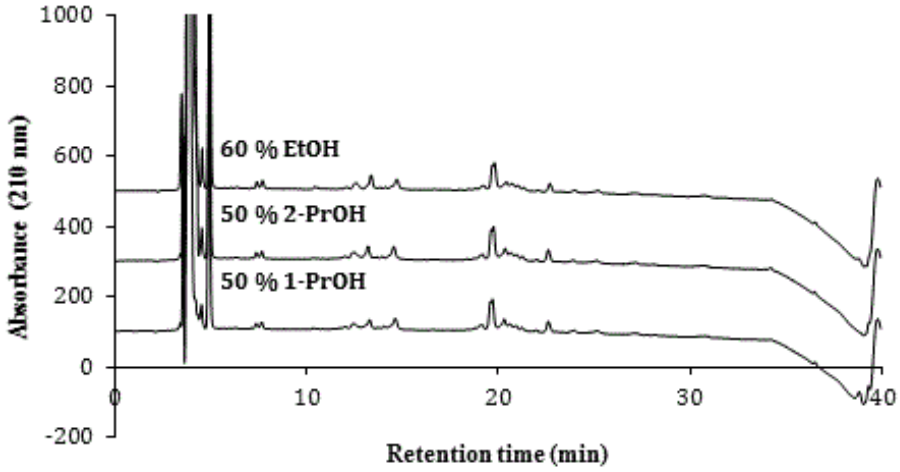
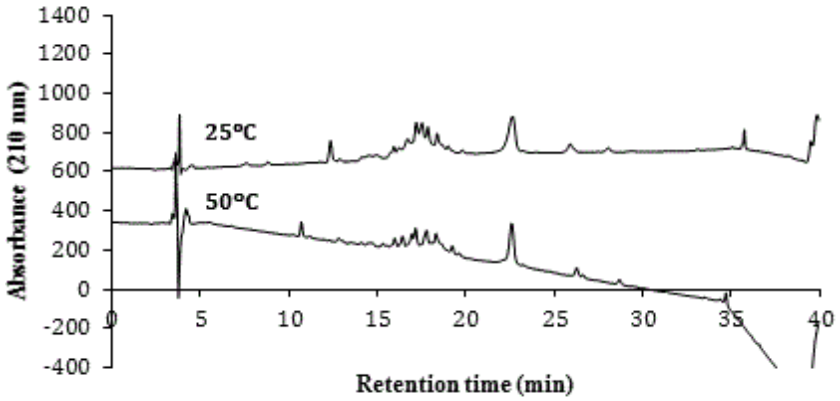


Figure 7. Reverse-phase liquid chromatograms of a) gliadin and b) glutenin extracts from wheat cultivar Julius (SWE) separated on a C8 column (50°C). Solvents used in the extraction: 60 % EtOH, 50 % 2-PrOH and 50 % 1-PrOH. For glutenin extraction also 5 % 2-mercaptoethanol was used.

5.1.2 Temperature

Gliadin and glutenin extracts were analysed at 25°C and 50°C in RP-HPLC to investigate the most suitable temperature for separation. To improve glutenin extraction, 50% 1-propanol (v/v) with 2 mol/l of urea, 0.05 mol/l of Tris-HCL (pH 7.5) and 1% (w/v) DTT was used. The retention times of the chromatograms with different temperatures used were different and also the baseline was different (Figure 8). For example with the gliadin sample at 25°C there can be seen a peak at ~12 min, whereas at 50°C, the same peak can be seen at ~10 min. The baseline of the glutenin chromatogram declined, and so did the baseline of 50°C gliadin chromatogram. With this solvent, glutenin separation was better, but the absorbance of the peaks was still low. In the following HPLC experiments, 50°C temperature is to be used with both gliadin and glutenin separation.

A



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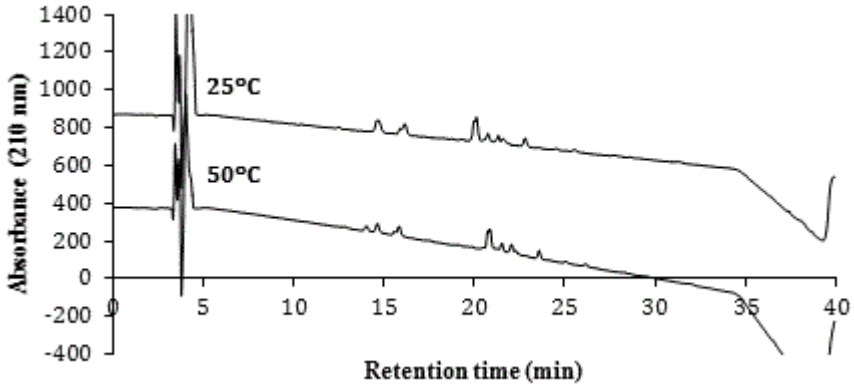
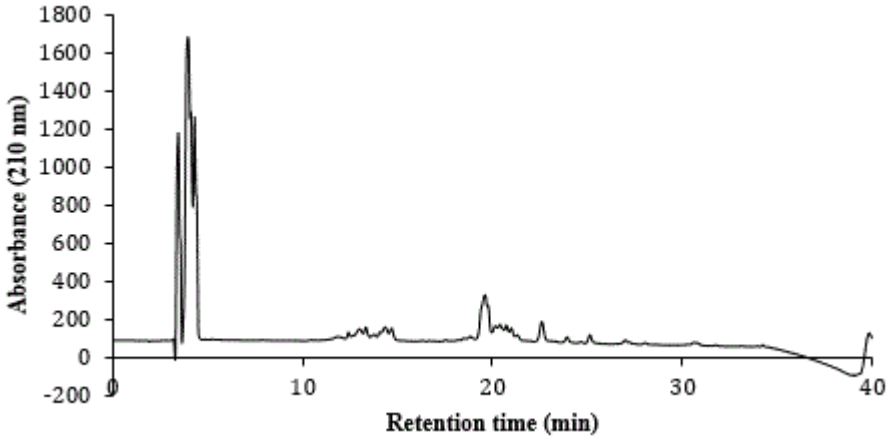


Figure 8. Reverse-phase liquid chromatograms of a) gliadin and b) glutenin extracts from wheat cultivar Crusoe separated on a C8 column, at 25°C and 50°C.

5.1.3 Glutenin injection volume

Glutenin extract was analysed by RP-HPLC with injection volumes 25 µl and 50 µl to investigate if higher injection volume would result in a better separation. Bigger injection volume resulted in better separation (Figure 9). In the following experiments 50 µl injection volume is to be used for glutenin HPLC separation. The void peak was still quite tall, which could mean that some of the glutenin subgroups were not properly solubilized.

A



B

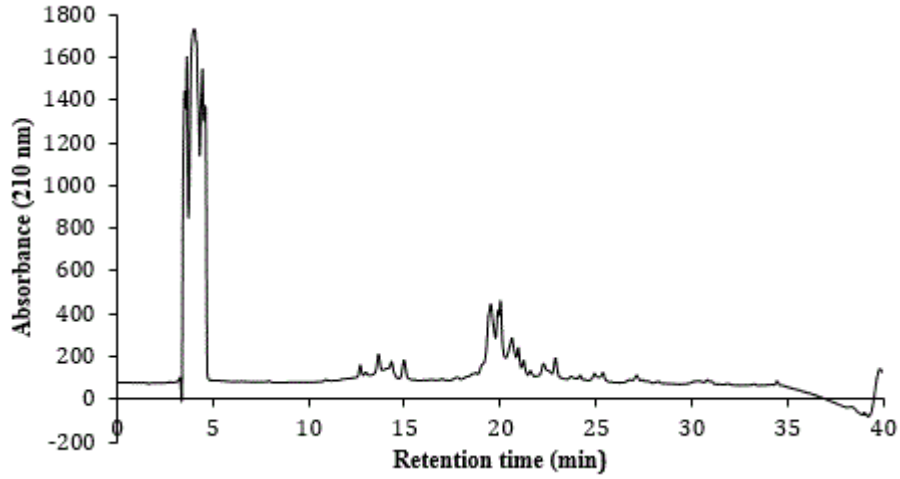


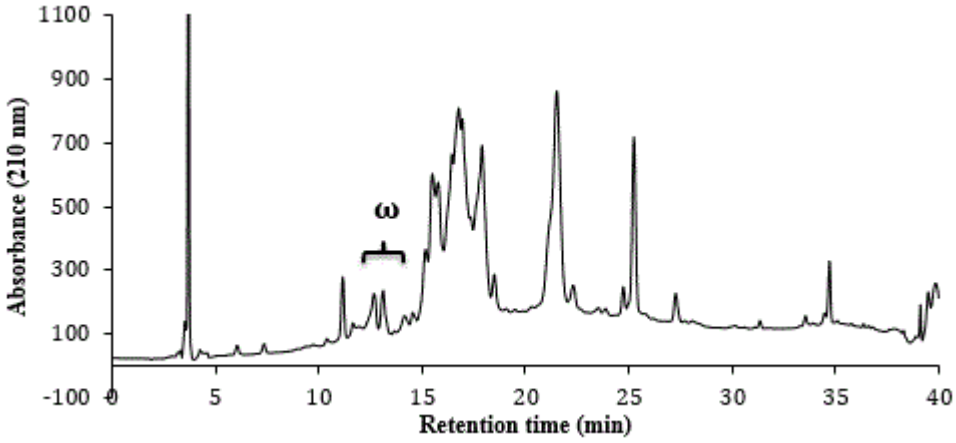
Figure 9. Reverse-phase liquid chromatograms of glutenin extract from wheat cultivar Crusoe separated on a C8 column (50°C). Injection volume a) 25 µl and b) 50 µl.

5.1.4 Gradient

Gliadin extract was analysed with two different gradients to investigate if a lower percentage of acetonitrile in the mobile phase would result in a better separation of ω-gliadin. This was the hypothesis since ω-gliadin is more hydrophilic than other gliadins. When whole gliadin was separated on a C8-column, the ω-gliadin was eluted first, followed by α/β-gliadins and γ-gliadins. The lower percentage of acetonitrile resulted in a smaller void and taller peaks for ω-gliadin in the chromatogram (Figure 10). In addition, the retention times of ω-gliadin were different between the different gradients. The gradient with higher percentage of acetonitrile resulted in a retention time of 13 min whereas the gradient with lower percentage of acetonitrile resulted in 18 min retention

time. Only cultivar Crusoe was analysed with the different gradients, whereas other cultivars were analysed in HPLC with gradient A. The fractionation of cv. Crusoe was decided to conduct using the gradient B.

A



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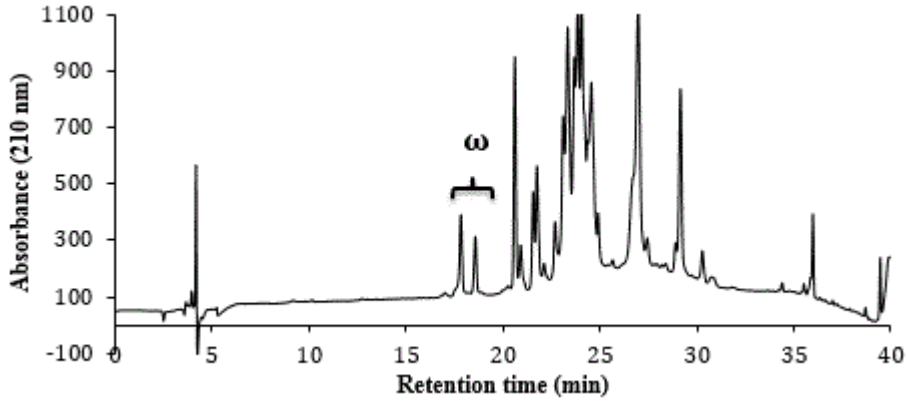


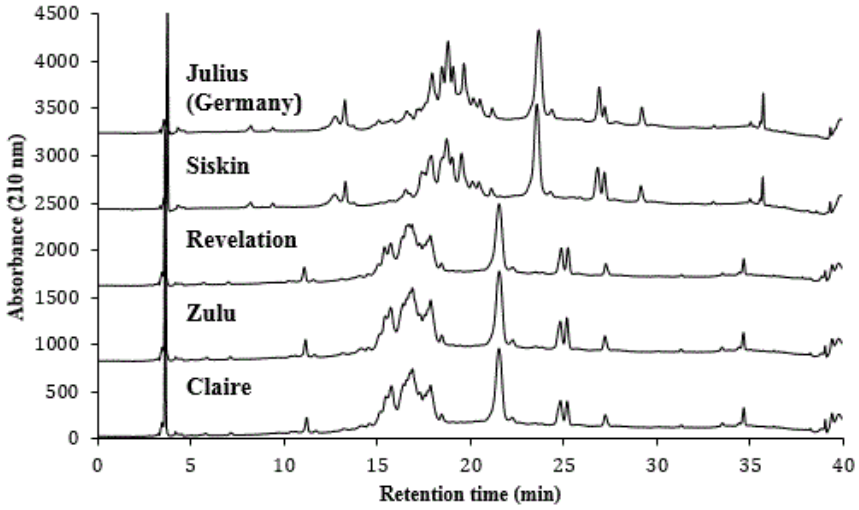
Figure 10. Reverse-phase liquid chromatograms of gliadin extract from wheat cultivar Crusoe separated on a C8 column (50°C). Gradients: a) 0 min 28 % B, 30 min 56 % B, b) 0 min 15 % B, 30 min 60 % B. ω: ω-gliadin.

5.2 Prolamin compositions of different wheat cultivars

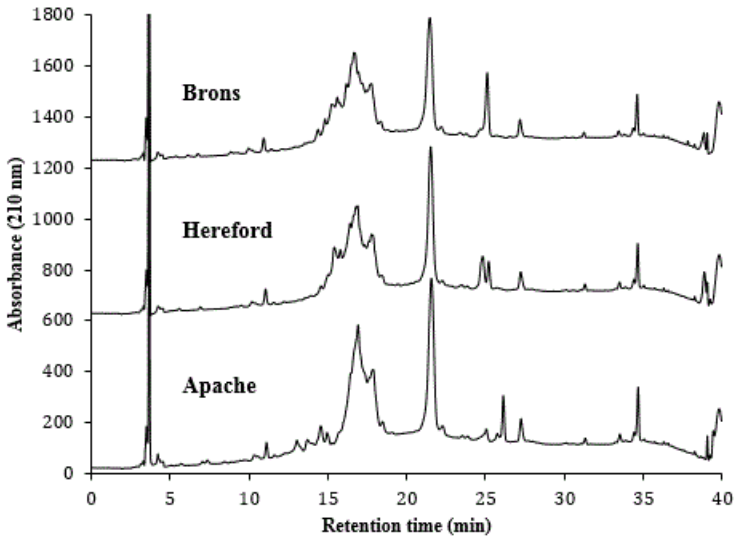
5.2.1 HPLC gliadin profiles

Wheat prolamin HPLC profiles of 16 analysed cultivars were grouped into four groups based on similarities on their gliadin peak patterns (Figure 11). The most common peak pattern was type D and it was present in 6 cultivars. All the groups had a tall, singler peak at 22-23 min. For the third group (cultivars Suntop and Lancer) the peak was the tallest of all the cultivars (Figure 11 C). All the cultivars showed several peaks at 15-20 min. At 10-15 min, all the cultivars showed smaller, single or double peaks.

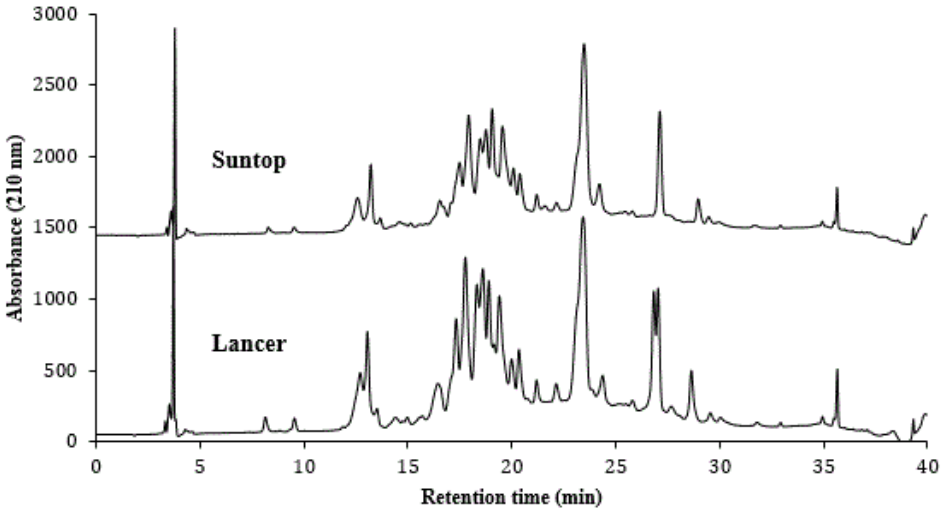
A



B



C



D

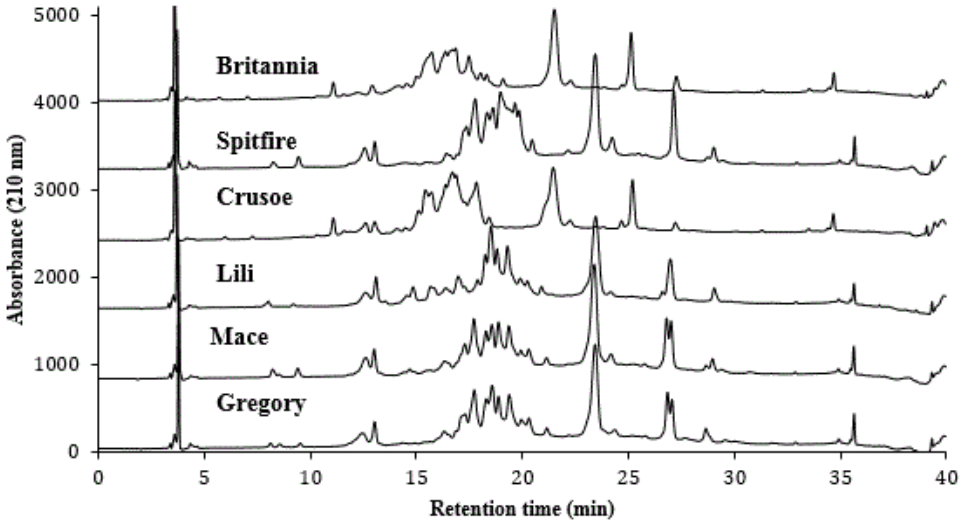


Figure 11. Reverse-phase liquid chromatograms of whole gliadin extracts from wheat cultivars (A) Julius, Siskin, Revelation, Zulu and Claire; (B) Brons, Hereford and Apache; (C) Suntop and Lancer; (D) Britannia, Spitfire, Crusoe, Lili, Mace and Gregory. All cultivars were separated on a C8 column (50° C).

5.2.2 ω-gliadin proportions in total gliadin

The ω-gliadin peaks were manually integrated and the peak areas were compared to determine the ω-gliadin composition of total gliadin in cultivars that were selected to analyse in RP-HPLC (Table 6). The ω-gliadin proportions between different cultivars varied greatly. For example, the ω-gliadin proportion in total gliadin of cv. Julius was 14.1 % whereas in cv. Hereford it was 0.8 %, after Osborne extraction. The ω-gliadin proportions varied also between the cultivars of same group (similar gliadin peak pattern). For example, cv. Julius had richly ω-gliadin in comparison to cv.

Revelation from the same group A, which had 5.5 % ω-gliadin in total gliadin. Cultivars from the same origin had smaller differences between their ω-gliadin proportions. Cultivars from Australia (Suntop and Lancer) had ω-gliadin proportions from 9.2 to 10.4 % and cultivars from Sweden (Brons and Hereford) had ω-gliadin proportions from 0.8 to 1.2 %.

Table 6. ω-gliadin content as a percentage of whole gliadin of selected wheat cultivars.

Group	Cultivar	Country	Content %	Group	Cultivar	Country	Content %
A	Julius	Germany	14.1	C	Suntop	Australia	9.2
	Siskin	UK	7.8		Lancer	Australia	10.4
	Revelation	UK	5.5	D	Britannia	UK	10.1
	Zulu	UK	6.7		Spitfire	Australia	4.6
	Claire	UK	6.1		Crusoe	UK	11.8
B	Brons	Sweden	1.2		Lili	UK	9.2
	Hereford	Sweden	0.8		Mace	Australia	9.8
	Apache	France	13.9		Gregory	Australia	5.2

5.2.3 The use of reducing agent in SDS-PAGE

The SDS-PAGE of whole gliadin of cultivars Julius (GER), Brons, Hereford, Apache, Britannia, Claire, Zulu, Revelation and Crusoe was carried out with and without reducing agent (5% 2-mercaptoethanol) in the SDS-buffer. ω-gliadin was observed at ~80 kDa and α/β- and γ-gliadin at ~40 kDa (Figure 12). The gel with added 2-mercaptoethanol showed protein at ~110 kDa whereas in the one without the reducing agent the protein could not be seen. The protein showing at ~110 kDa was probably HMW-GS, although these samples were extracted by Osborne extraction for gliadins. The size is equal to the size of the glutenins showing in the SDS-PAGE figures in following results.

Part of the standard and the samples of lower molecular weights was not showing, because this gel was advised to be run in SDS-PAGE only for 30 min, instead of 50 min. However anything important was not missing and the gels were successful, so 40-50 min run time was decided to be used with this gel also in the following experiments.

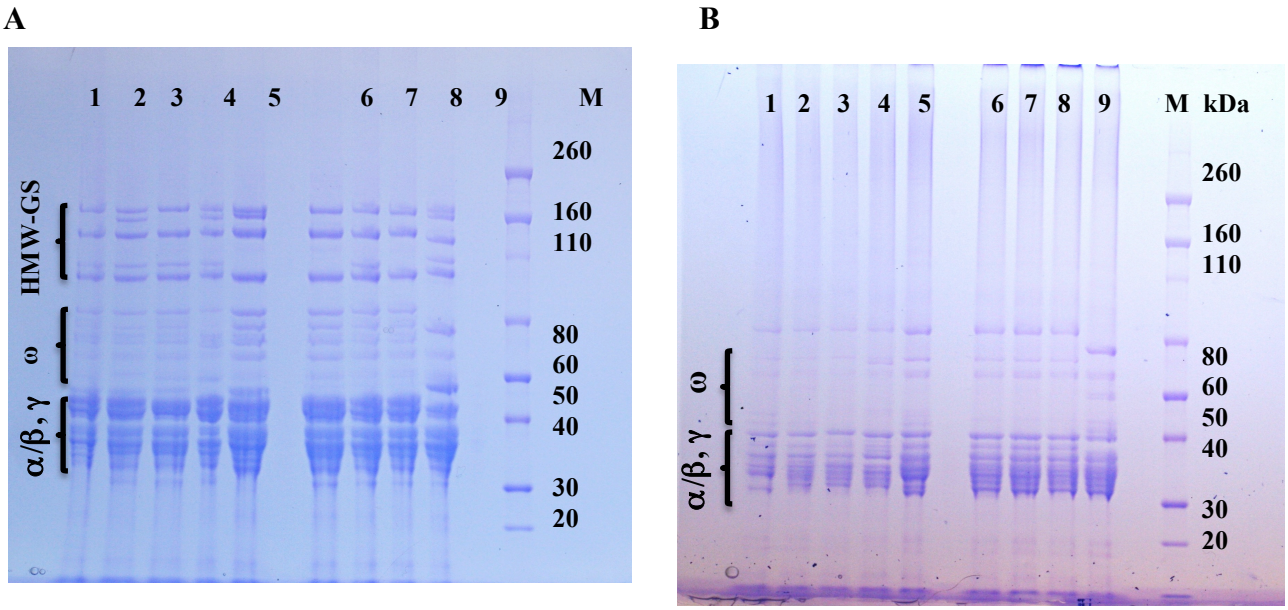


Figure 12. SDS-PAGE of gliadin extracts (A) with and (B) without 2-mercaptoethanol (5 %). Cultivars: 1) Julius (GER), 2) Brons, 3) Hereford, 4) Apache, 5) Britannia, 6) Claire, 7) Zulu, 8) Revelation, 9) Crusoe. M: marker, ω: ω-gliadin, α/β: α/β-gliadin, γ: γ-gliadin. Load volume 8 µl.

5.2.4 SDS-PAGE of gliadin and glutenin extracts

SDS-PAGE was carried out to whole gliadin and glutenin extracts of all the wheat cultivars. For all the cultivars the run time of SDS-PAGE was 50 min, but with the cv.1-13 10% Bis-Tris-Gel was used whereas with the cv. 14-21 4-12% Bis-Tris Plus gel was used (Figure 13). For the latter, a run time of 30 min would have been the recommended run time, however all the gels were successful. ω-gliadin was observed at ~80 kDa and α/β- and γ-gliadin at ~40 kDa (Figure 13 A). HMW-GS was observed at ~110 kDa and LMW-GS at ~40 kDa (Figure 13 B). From these gels the differences between different cultivar prolamin compositions can be seen. For example whole gliadins of cv. Lili (16) and Suntop (17) show different lines in the gel and different prolamin subgroups are present as different volumes depending on the cultivar. The load volumes of the samples varied. For example cv. Crusoe was loaded with load volumes of 8, 4, 2 and 1 µl (1-4). The smaller load volumes can be seen as fainter lines in the gel. As previously, also here there can be seen some HMW-GS in the gels of whole gliadin at ~110 kDa.

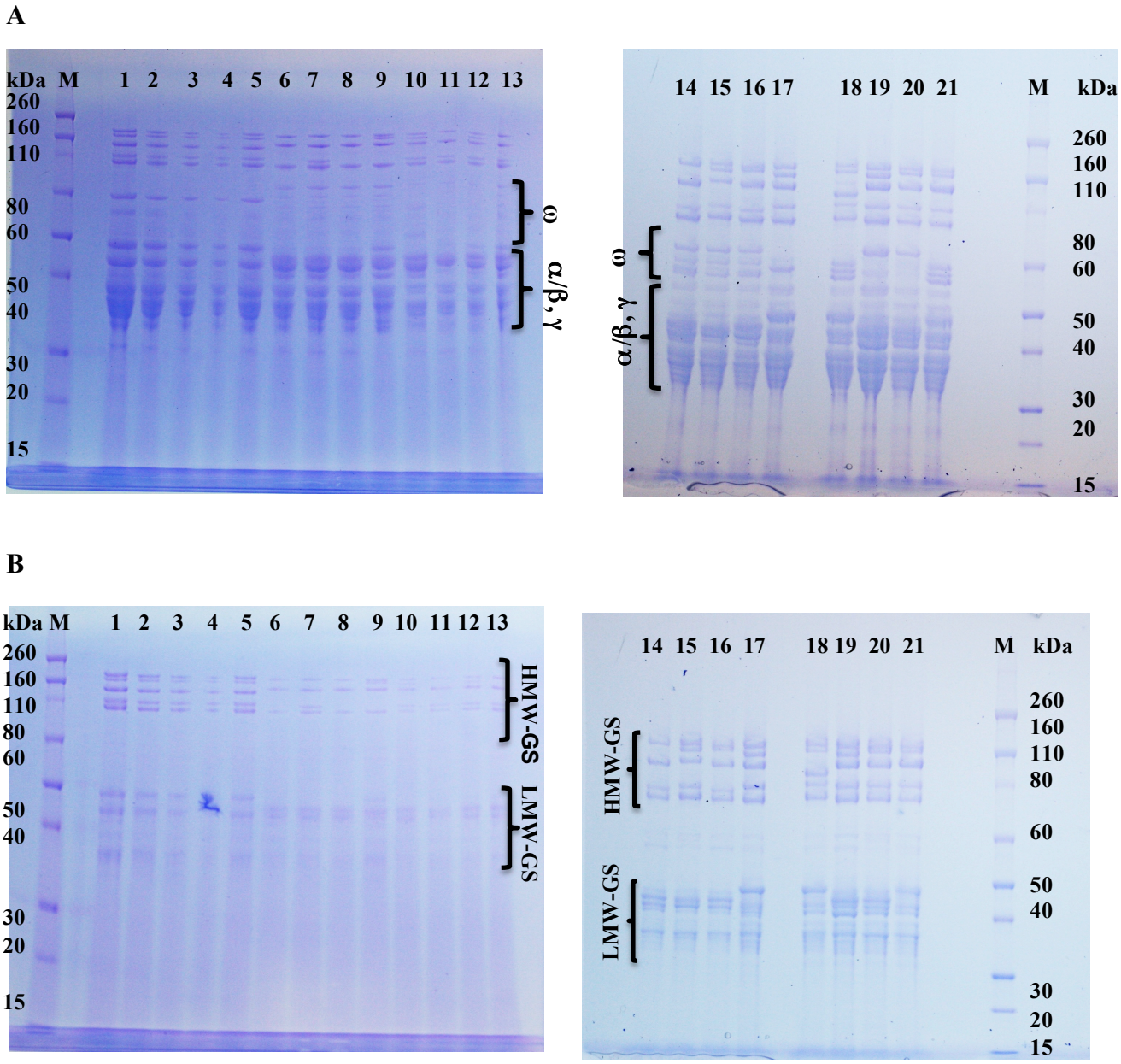


Figure 13. SDS-PAGE of (A) gliadin and (B) glutenin extracts. Cultivars: 1-5) Crusoe, 6) Revelation, 7) Zulu, 8) Claire, 9) Britannia, 10) Apache, 11) Hereford, 12) Brons, 13) Julius (SWE), 14) Julius (GER), 15) Siskin, 16) Lili, 17) Suntop, 18) Spitfire, 19) Lancer, 20) Gregory and 21) Mace. M: marker, ω: ω-gliadin, α/β: α/β-gliadin, γ: γ-gliadin. For cultivars 1-4 load volumes are 8, 4, 2 and 1 ul, for 5-13 4 ul and for 14-21 8 ul.

5.2.5 SDS PAGE of whole gluten

SDS-PAGE was carried out to whole gluten extracts of all the cultivars. HMW-GS was observed at ~60-100 kDa and LMW-GS at ~30-40 kDa. In addition, ω-gliadin was observed at ~50-60 kDa and α/β- and γ-gliadin at ~30-40 kDa (Figure 14). The staining of the gels was excellent and the bands could easily be seen. The load volume was same for all the samples, so the differences in prolamin

composition between different cultivars could be compared. For example for cv. Crusoe (2), at the range of ω-gliadin 3 bands can be seen, whereas for cv. Julius (1) and Revelation (3), 4 bands can be seen at the same range. It indicates that cv. Julius and Revelation have some additional gliadin-sub-units in comparison to cv. Crusoe. Also the thickness of the bands is varying between the cultivars. Some cultivars are also quite similar. For example cv. Kerubino (19) and Patras (20) have similar gluten patterns.

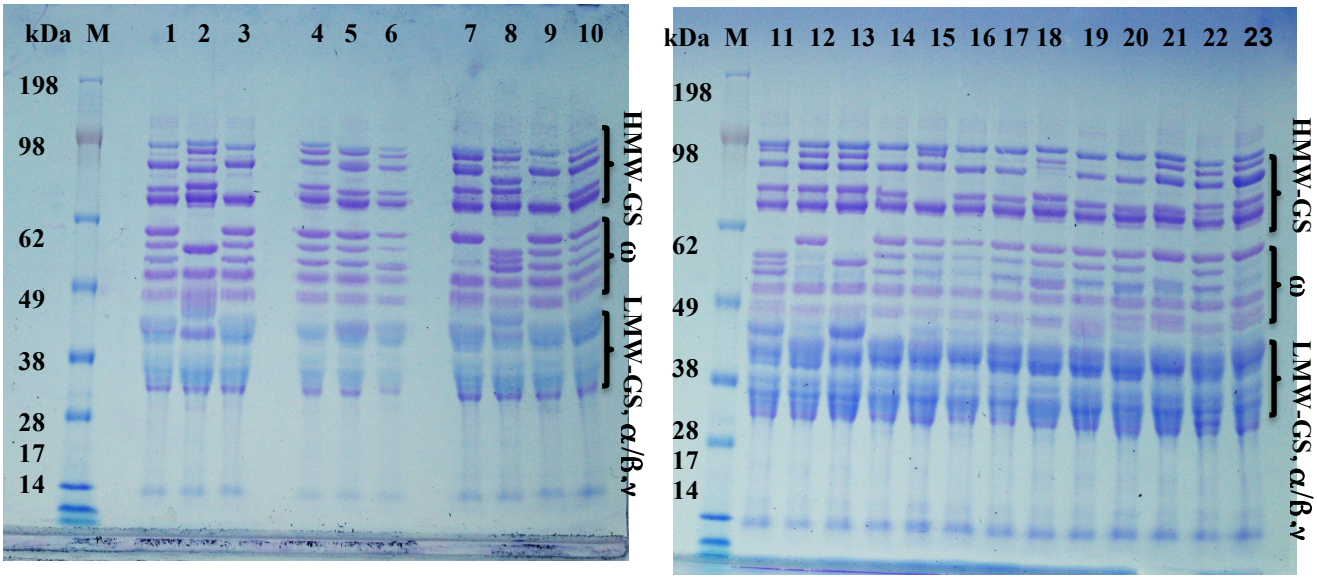


Figure 14. SDS-PAGE of gluten extracts of different cultivars. Cultivars: 1) Julius (GER), 2) Crusoe, 3) Revelation, 4) Siskin, 5) Lili, 6) Apache, 7) Gregory, 8) Spitfire, 9) Claire, 10) Brons, 11) Mace, 12) Lancer, 13) Suntop, 14) Zulu, 15) Britannia, 16) Hereford, 17) Julius (GER), 18) Cellule, 19) Kerubino, 20) Patras, 21) Amaretto, 22) Anniina and 23) Quarna. M: marker, ω: ω-gliadin, α/β: α/β-gliadin, γ: γ-gliadin. Load volume 3 ul.

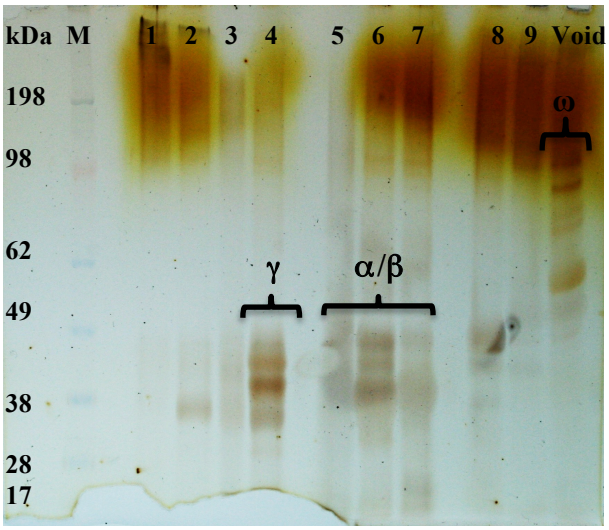
5.3 Characterization of prolamin groups by RP-HPLC fractionation and SDS-PAGE

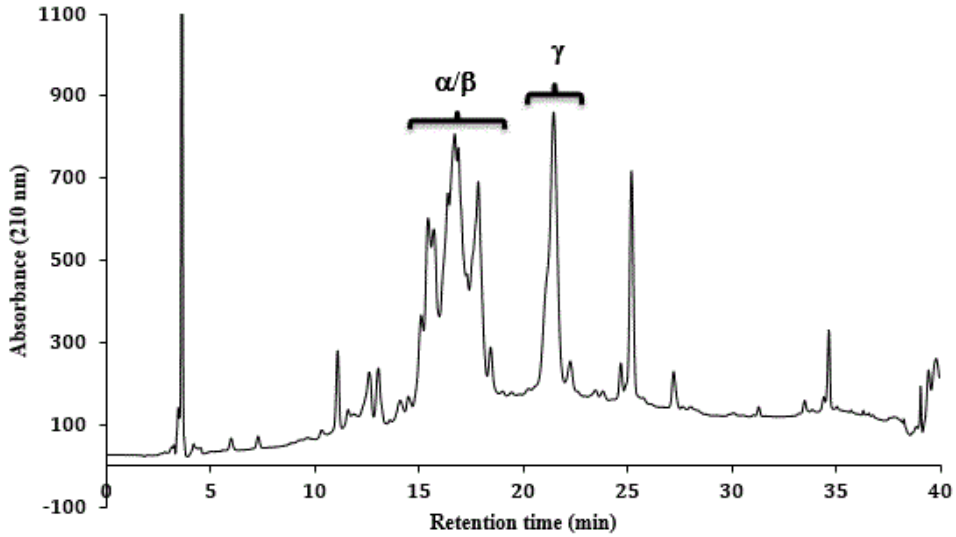
The fractions of RP-HPLC whole gliadin and glutenin peaks of cv. Crusoe were collected and SDS-PAGE was carried out to identify the fractions. The silver staining of the gels was poor and the background of the gels was partly stained too. Prolamins were separated into the following groups: ω-, α/β- and γ-gliadins. Glutelins were divided into following groups: HMW-GS and LMW-GS.

In the gliadin SDS-PAGE the ω-gliadins seemed to be showing only in the void peak, at ~60 kDa (Figure 15 A, 10). This indicates that the separation was not completely successful and some of the protein was in the void peak. SDS-PAGE indicated that peaks number 6 and 4 consisted of α/β- and γ-gliadins, which can be seen at ~40 kDa.

The glutenin SDS-PAGE shows that fractions number 8, 7 and 6 consisted of LMW-GS, which can be seen at ~40 kDa (Figure 15 B). The HMW-GS were showing in fractions number 9 and 10 at ~100 kDa.

A





B

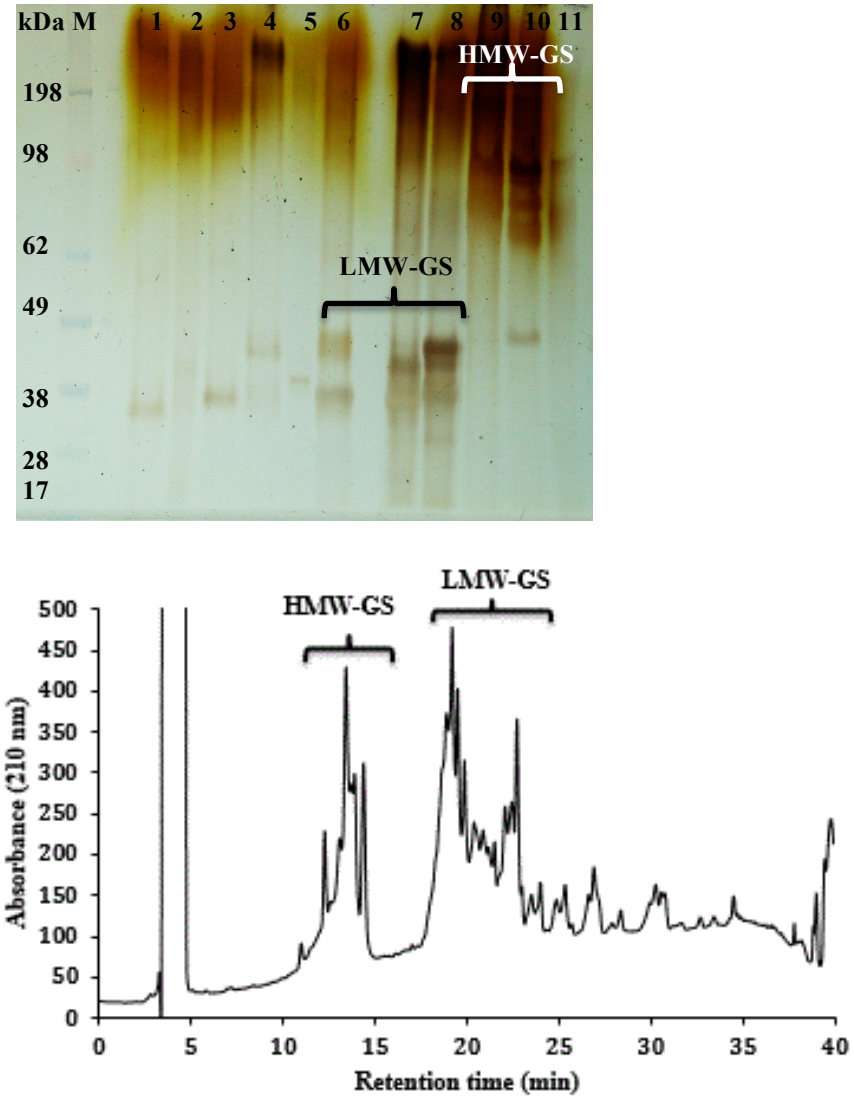
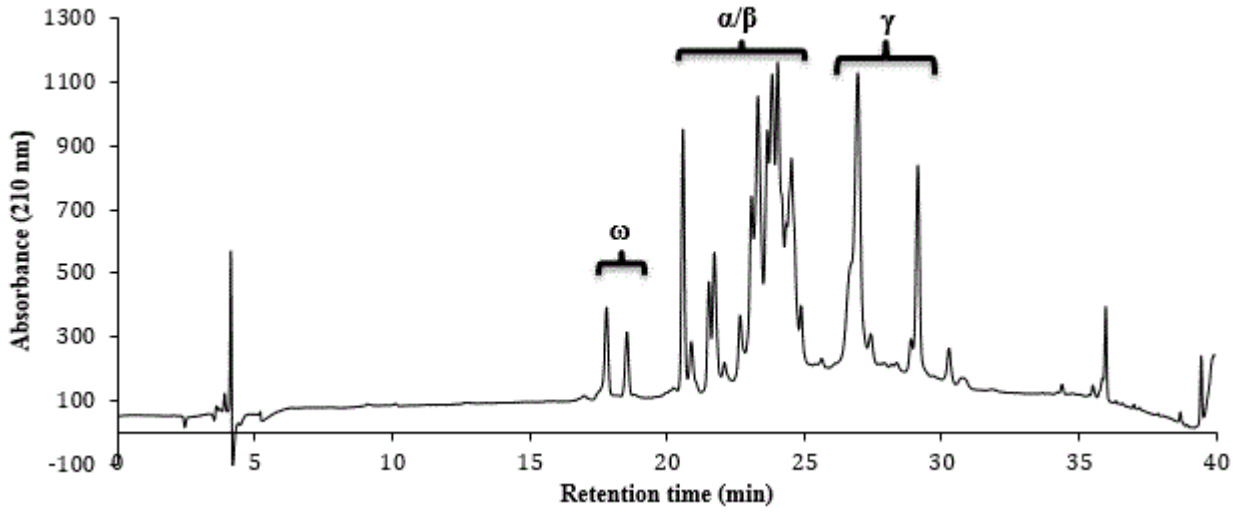


Figure 15. SDS-PAGE and reverse-phase liquid chromatograms of (A) gliadin and (B) glutenin extracts from cultivar Crusoe, separated on a C8 column (50°C). The numbers in the SDS-PAGE are indicating the collected fractions. M: marker. Gradients: 0 min 28% B, 30 min 56% B. SDS-PAGE load volumes are 12 ul, except for number (A) 3 (3 ul) and (B) 2 (5 ul), 6 (5 ul) and 11 (4 ul).

Also another two sets of cv. Crusoe's gliadin fractions were collected, with following linear acetonitrile gradients: 1) 0 min: 15 % B, 30 min: 60 % B and 2) 0 min: 20% B, 30 min: 60% B. The gradient with less acetonitrile than previously was assumed to promote better separation of ω-gliadin separation since ω-gliadin is hydrophilic. There was no difference in the separation of these two gradients. The presented figures are from the separation with the prior gradient. The samples were rereduced and alkylated before the SDS-PAGE.

The silver staining of the gel was yet poor and the staining of the background made the analyzing of the gel difficult. With lower acetonitrile content in comparison to the previous results, ω-gliadin was separated. SDS-PAGE of the collected fractions ω-gliadins, α/β-gliadins and γ-gliadins could be identified (Figure 16). The protein content was low, thus also the bands were faint. However, ω-gliadin could be identified as a very faint band at ~80 kDa and α/β- and γ-gliadin at ~40 kDa.

A



B

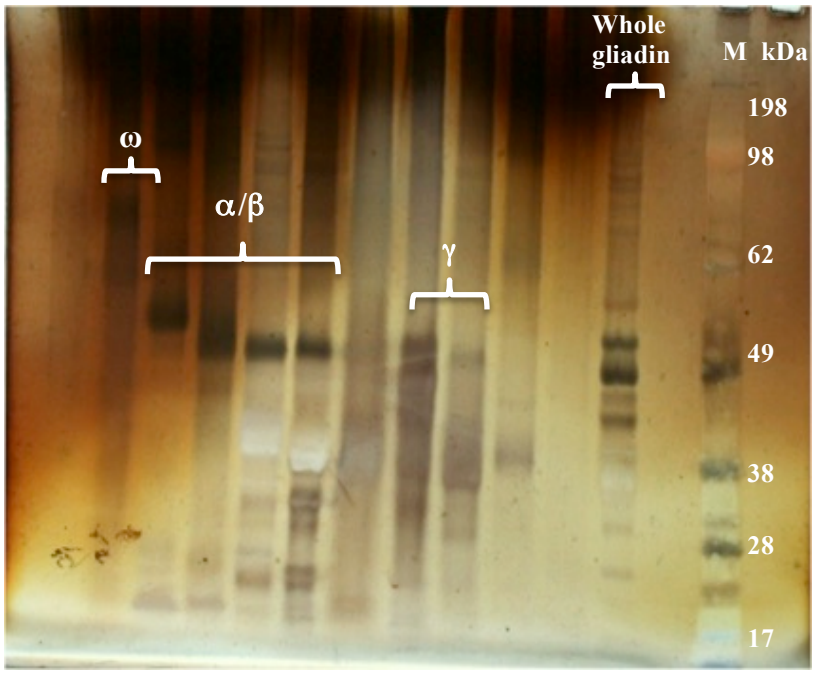


Figure 16. (A) Reverse-phase liquid chromatogram of gliadin extract from wheat cultivar Crusoe, separated on a C8 column (50°C). Gradients: 0 min 15% B, 30 min 60% B. Fractions from peaks were collected. (B) SDS-PAGE of collected fractions. SDS-PAGE sample load volumes were 15 µl.

5.4 C-hordein as reference material for wheat gluten quantification

5.4.1 Protein contents of the extracted wheat gluten samples by Dumas

Protein content of the extracted gluten of all the analysed cultivars was determined by Dumas method (Table 7). The protein contents were in the range of 74-90 %. Total gluten was extracted only once, thus all of the gluten was probably not extracted.

Table. 7. Protein content of the extracted whole gluten of each cultivars, determined by Dumas method. Mean standard deviation could not be determined due to the small sample size.

Wheat cultivar	Protein content of extracted whole gluten %	Wheat cultivar	Protein content of extracted whole gluten %
Brons	76	Anniina	83
Claire	83	Patras	81
Spitfire	86	Lancer	90
Gregory	83	Cellule	84
Apache	77	Suntop	86
Lili	84	Britannia	78
Siskin	80	Quarna	83
Revelation	78	Kerubino	83
Crusoe	85	Zulu	86
Julius (Germany)	82	Julius (Sweden)	76
Amaretto	85	Hereford	74

5.4.2 Wheat prolamin reactivity against R5 antibody

The wheat prolamins of different cultivars, C-hordein with BSA in different ratios (10, 20 and 30%) and gliadin standard were investigated against R5 antibody in sandwich ELISA. The cultivars presented in the figure (Julius, Hereford, Lancer and Crusoe) were selected from each gliadin peak pattern group (A, B, C and D, respectively). The response of the ELISA systems antibody to increasing prolamin concentration was plotted on a log axes (Figure 17). The slope of the curve of hordeins with R5 antibody had a similar behavior to gliadin standard. The reaction of 30% C-hordein was the most similar with the gliadin standard. The curves of purified whole gluten of cv. Hereford and Julius were similar with each other and with 20% C-hordein, although they had different proportions of ω-gliadin (0.8% and 14.1%, respectively). For cv. Crusoe (ω-gliadin proportion of 11.8%) the curve was above them and more similar with the gliadin standard and 30%

C-hordein. The curve of cv. Lancer (ω-gliadin proportion of 10.4%) was above of all the hordeins and the gliadin standard. The curves did not reach a flat stage, which indicates that more dilution points would have been needed.

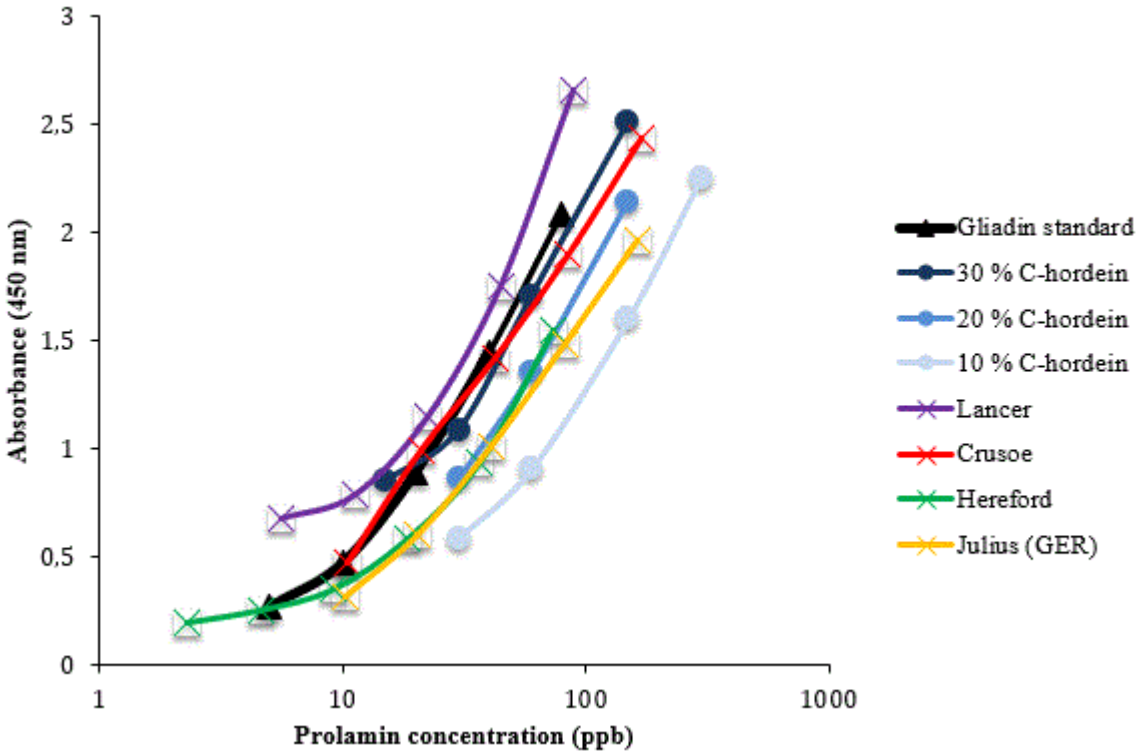


Figure 17. Reaction of wheat prolamins (cultivars Hereford, Crusoe, Julius and Lancer), C-hordein (10%, 20% and 30% extracts) and gliadin standard against R5 antibody in sandwich ELISA.

The Km values of prolamins were determined from the curves (Table 8). The Km is a measure of the amount of prolamin required to produce a half-maximal response in the ELISA assay. In other words, it is a measure of sample sensitivity in the assay. The lower the value, the more sensitive was the detection. The Km-values for the different cultivars were not as expected. The cultivars with high ω-gliadin content were not highly reactive. For example Swedish cv. Julius had ω-gliadin proportion of 14.1 %, but the reactivity was 118, which is higher than average of the selected cultivars. For cv. Hereford, the ω-gliadin proportion in total gliadin was only 0.8 %, but here the Km-value was 79, thus it was more reactive than cv. Julius. The C-hordein samples in different ratios resulted in Km-values as expected. The higher the proportion of C-hordein was in the sample, the lower was also the Km-value.

The average Km value of all the cultivars was 91.8. For 20% C-hordein the Km value was 90.3, whereas for gliadin standard it was 67.8. This indicates that for these selected cultivars, the 20% C-hordein was the closest one in reactivity from these standard materials. Thus, the 20% C-hordein was the best reference material for these samples, since a good reference material is as close in reactivity to the real samples as possible.

Table 8. Comparison of Km of prolamins. The data were replotted as linear Michaelis-Menten plots, and the Km values determined from the curves of best fit (GraphPAD Prism 6).

Sample	**Km ± Std. error**	**Sample**	**Km ± Std. error**
Lili	65 ± 14	Quarna	67 ± 20
Siskin	78 ± 9	Cellule	59 ± 3
Revelation	74 ± 4	Julius (SWE)	118 ± 42
Crusoe	51 ± 6	Hereford	79 ± 27
Julius (GER)	78 ± 3	Britannia	94 ± 19
Apache	103 ± 45	Zulu	23 ± 7
Brons	275 ± 86	Lancer	51 ± 20
Claire	84 ± 9	**Cultivar average**	92
Spitfire	100 ± 31	**10 % C-hordein**	169
Gregory	143 ± 50	**20 % C-hordein**	90
Amaretto	110 ± 10	**30 % C-hordein**	59
Anniina	82 ± 28	**Gliadin standard**	68
Kerubino	103 ± 16		

6 DISCUSSION

6.1 Method optimization

In the preliminary tests it was found that there was no difference whether the prolamins were extracted with 1-propanol (50% v/v), 2-propanol (50% v/v) or ethanol (60% v/v), as they all resulted in similar separation in HPLC. In previous studies, the most efficient solution for extracting wheat, barley and rye prolamins has found to be 40% 1-propanol (Kanerva et al. 2011).

In preliminary tests, SDS-PAGE was conducted with and without reducing agent. Without reducing agent, HMW-GS could not be detected in the gel. This could be due to the fact that gluten proteins aggregate easily, because of formation of disulfide bonds (Diaz-Amigo and Yeung 2010). Breaking the bonds by reducing agent is necessary for the solubilization of prolamins) and thus for the large proteins to show in SDS-PAGE (Wagner et al 2011). Another aspect of this result is that, it indicates that the solubility of the prolamin classes is not absolute. Also previous studies have shown that there is a possibility that a salt-water extract of flour contains gliadin and an ethanol extract contains HMW-glutenin although they would not follow Osborne solubility classification (Fu and Sapirstein 1996; Hamer 2003). Additionally, it has to be taken into account that an ethanol extract of flour will not contain all the gliadins of the sample, and a salt water extract will not contain all the albumin and globulin proteins. This is a concern when an assay is used for quantification. Generally, aqueous alcohol solutions have been used in gluten detection methods under the consideration that they extract the prolamin fraction alone (Diaz-Amigo and Yeung 2010). However, as shown also in this study, the aqueous alcohol solutions without any reducing agents can also extract glutenins.

All ELISA protocols aim to quantify the total gluten content in a food sample, but at the same time full extraction of all gluten proteins is problematic due to differences in the solubility of gliadin and glutenin (Diaz-Amigo and Popping 2013). The advised extraction protocols vary between commercial ELISA kits, as they include proprietary extraction buffers and different incubation times and temperatures (Rzychon et al. 2017). In this study, the extraction of gliadin and glutenin

was successfully improved by investigating different solvents. The whole gluten samples used in ELISA were extracted using the cocktail-solution from the kit.

The whole gliadin extract of cv. Crusoe was analysed with lower percentage of acetonitrile in the mobile phase (0 min 15% B, 30 min 60% B) in order to investigate if it would result in a better separation of ω-gliadin, because of its hydrophilicity. It resulted in improved ω-gliadin separation. In previous studies the linear gradients for HPLC gliadin and glutenin separation was 0 min 28% B, 30 min 56% B (Wieser et al. 1998) and 0 min 0% B, 0,5 min 24% B, 20 min 56% B, 20,1-24,1 min 90% B, 24,2-30 min 0% B (Schalk et al. 2017). In the study by Schalk et al. (2017) the ω-gliadins were well separated and also here the gradient was low in acetonitrile in the beginning. Also in this study it would have been interesting to see the separation with a gradient that has 0% of acetonitrile in the beginning.

The crude protein contents of whole gluten samples were between 74 and 90%. The statistical analysis could not be conducted since the sample amount was too low for replicates. In previous studies the crude protein content for gliadins have been 93.5% and for glutenins 82.8% (Schalk et al. 2017). This shows that the extraction procedure from the wheat flours followed by dialysis yielded gluten extracts with rather high protein contents for only one time extraction. For PWG-gliadin, protein content determined by Dumas has been reported to be 89.4% (van Eckert et al. 2006). In comparison to these results, the protein content is comparable, although slightly lower. Higher crude protein content could have been achieved by repeating the extraction procedure. In addition, defatting of the samples would result in higher protein content of the samples. The protein contents of the gluten fractions were not determined as in the study of Schalk et al. (2017), nor was their identity confirmed by re-chromatography. Thus the purity of the gluten fractions was not confirmed. Also, in previous studies the isolation of gluten protein fractions has been done to defatted sample flours, which is advisible for especially oat samples (Schalk et al. 2017; Pflaum et al. 2013).

6.2 Prolamin compositions of the wheat cultivars

Subunits of the gliadin and glutenin fractions can be estimated based on order of elution, peak shape and peak height (Alvarez et al. 2013). Peaks between 15 and 20 min are HMW-glutenins and between 20-25 min LMW-glutenins (Wieser 2000). Two smaller peaks between 10 and 15 min are ω-gliadins, taller peak at 15-20 min α-gliadin and γ-gliadin as a single tall peak with small tapering peaks between 20-25 min. (Wieser et al. 1998).The qualitative RP-HPLC prolamin peak profiles correrssponded well to those reported in earlier studies (Schalk et al. 2017). It has been reported that ω-gliadins elute first, followed by α/β-gliadins and finally elute γ-gliadins. All wheat prolamin subgroups could be assigned for cv. Crusoe. Gliadins were separated into the following groups: ω-, α/β- and γ- gliadins. Glutenins could be separated into HMW- and LMW-glutenins. As stated previously, traces of HMW-GS were observed in the gliadin fraction SDS-PAGE. Perhaps they are in the void peak in the chromatograms.

Much variation was found to occur in the gluten subunit banding patterns of different cultivars, which has been seen also in previous studies (Lawrence and Shepherd 1980). Also the thickness of the bands is varying between the cultivars. The characteristic bands for each gliadin and glutenin subunits were observed at the MW ranges of 80-110 kDa for HMW-GS, 50-62 kDa for ω-gliadins and 32-45 kDa for α/β-, γ-gliadins and LMW-GS. They correspond to the results of previous studies (Lagrain et al. 2012). The ω5- and ω1,2-gliadins could not be separated in this study, hence the ω-gliadins were observed in one group. In literature, the MW ranges for ω5-gliadins have been reported to be 60-68 kDa and for ω1,2-gliadins 43-60 kDa (Lagrain et al. 2012). For other cultivars, the fractions of gliadins were not collected. However, the chromatograms corresponded well to those of wheat gliadins in previous studies, hence estimates about the identity of the peaks could be drawn (Schalk et al. 2017).

The silver staining of the SDS-PAGE of the prolamin fractions was poor. The staining was conducted several times with varying staining times or the temperature of the reagents, but the background of the gels was always partly stained making the reading of the gels difficult. In addition, the proportion of protein in the sampe was low in the fractions, thus also the bands of ω-gliadin were faint. However, also the SDS-PAGE confirmed the finding that with the lower acetonitrile content in the mobile phase of the HPLC, the ω-gliadins were separated. In the SDS-

PAGE with the higher proportion of acetonitrile in the mobile phase the ω-gliadins can clearly be seen in the void peak.

The proportion of ω-gliadin in total gliadin ranged from 0.8 to 14.1% between the selected cultivars. It has to be taken into consideration that some of the prolamins always remain unextracted. Nevertheless, the ω-gliadin proportions of wheats from the same origin had similarities, and it is known that the gliadin composition is dependent on soil and growing conditions of wheats (Grain prolamin composition are highly affected by the growing conditions (Kirkman et al. 1982; Wieser and Kieffer 2001). For example wheats from Sweden had the lowest proportions of ω-gliadin (0.8-1.2%), whereas the wheats from Australia had ω-gliadin from 4.6 to 10.4%. Also in previous studies the crude protein content and the proportion of gliadin subgroups between wheat cultivars have varied greatly (Hajas et al. 2017). The gliadin composition has been reported to depend on the cultivar, but harvest year has been found to influence too because of yearly environmental variations.

In a previous study the ω-gliadin proportion was isolated from a mixture of four different wheat cultivars, thus the variation between different cultivars could not be seen (Schalk et al. 2017). The ω-gliadin proportion was reported as 19.8% in total gliadin. The ω-gliadin proportion in total gliadin has been reported to be between 6.2 to 20% (Wieser et al. 1994). In the PWG-gliadin the proportion of ω-gliadin in total gliadin has been reported as 11.3% by RP-HPLC analysis (van Eckert et al. 2006). The C-hordein proportion in whole hordein has been reported to vary from 16.5 to 33.1% in Finnish barley cultivars (Huang et al. 2017). In this study the quantitative values were in agreement when comparing to earlier studies, although the low proportion of ω-gliadin in the Swedish cultivars (Hereford and Brons) is questionable. In the chromatograms of Hereford and Brons there cannot be seen any peaks at the retention time where other cultivars had the ω-gliadin peaks. Therefore there is a possibility that the separation of ω-gliadin for these cultivars were not successful.

6.3 C-hordein as reference material for wheat gluten quantification

In addition previous studies had concluded that it was feasible reference material for barley gluten quantification (Huang et al. 2017). In addition to gliadins, R5 antibody has been found to recognize also glutenins (Kanerva et al. 2011). Also, it was found to recognize ω-gliadin and HMW-glutenin less intensively. Because the results are controversial, perhaps not everything is known about the antibody binding.

In gluten quantification, there should be a low risk of false acceptance, in order to avoid a risk of health issues to a person with coeliac disease. The recommendation is to provide at least 97.5% probability that the gluten concentration in a sample is less than the threshold (Eurachem/CITAC 2007). In a study by Rzychon et al. (2007) only 4 of the analysed 24 samples had the same classification in all of the assays. Based on scientific evidence it can thus be concluded that the current ELISA measurement cannot support the legislative requirements for gluten-free claims. The possible accuracy by individual kit assays sets the limit for harmonization of gluten measurements. The harmonization can only be improved by optimizing the analytical protocols and manufacturing processes. There was found to be 50% probability that a food product labeled gluten-free may contain up to 80-90 mg/kg gluten (Rzychon et al. 2007).

The dilution of the wheat gluten samples for ELISA was different for each cultivar, which could be due to the fact that they vary in reactivity against R5 antibody. Hence for each cultivar an optimal dilution had to be tested. As previously stated, the composition of gliadins from different cultivars was varying, and this was probably the reason that the whole gluten reactivity was also varying in the assay. The curves in ELISA did not reach the flat stage, where the higher concentration would have not changed the binding absorbance any more. Thus more dilution points would have been needed.

The prolamin contents achieved were not multiplied by a factor of two, thus from these results it could be seen how the reference materials represent the samples without any conversion factors. The use of conversion factor is questionable since the ratio of prolamin to glutelin varies (Lexhaller et al. 2016). In addition gliadins and glutenins, LMW glutenins in particular, share some amino

acid sequences, although only gliadins are assumed to react with R5 antibody (Diaz-Amigo and Popping 2013; Wieser 2007). For example QQPFP is this kind of amino acid sequence and it is also one of the R5 epitopes. Therefore reference material that would not need conversion factors would improve the method accuracy. Based on these results, it can be concluded that C-hordein can represent the whole gluten without using any conversion factors.

In this study, the 20% C-hordein reference material was the best fit for the selected wheat cultivars. Cultivars with the highest quantities of ω-gliadin were not the most reactive ones in the assay. Also the Swedish cv. Hereford with ω-gliadin proportion of only 0.8% in total gliadin was reported to be more reactive than many other cultivars. This could be due to the fact that the separation of ω-gliadin was not successful in the HPLC, as stated previously, but the sample in ELISA consisted of whole gluten. Thus it can be assumed that the reactive subgroups were present in the whole gluten sample. Contrarily to the results of this study, gliadin content measured with ELISA has been found to correlate positively with ω1,2- and α-gliadin proportion of flour (Hajas et al. 2017). The result was stronger with ω1,2-gliadin. Also van Eckert et al. (2010) has concluded that each antibody has been found to detect different gluten subgroups to a different degree. R5 antibody reacts strongly with ω-, γ- and α-gliadins and thus the reactivity of the sample is dependent on the proportion of these. According to these results the gluten amount detected was dependent on the gluten composition of the sample and the standard used (van Eckert et al. 2010). However, the diffusion of ω-gliadins out of the membrane during the incubation and assay washing was suggested to have diminished their response.

Kd-values are analogous to the Km-values presented in this study (Tanner et al. 2013). In previous studies the Kd-values has been reported to be 343 for commercial gliadin reference material, 84-212 for purified C-hordein of different cultivars and 64-670 for purified total hordein of different cultivars (Tanner et al. 2013). In this study the gliadin reference material resulted in higher reactivity, since the Km-value was 68. Based on the Km-values, the gliadin reference material was quite close to the reactivity of the selected cultivars, but it has to be taken into account that no conversion factors was used. Also, the 20% C-hordein was the closest one from the tested standards. The variation in reactivity between different wheat cultivars was in the same range with the previous studies of total hordein reactivity. It has been reported that the variation between

ELISA kits is due to the presence of different amounts of epitopes among the cultivars and their natural changes over the harvest years (Hajas et al. 2017).

Also batch-to-batch variability of the kits has been found (Rzychon et al. 2017). The variation of test results obtained from ELISA kits has been suggested to be due to differences in responses obtained for different types of samples, such as matrix or level of processing (Rzychon et al. 2017). In this study the sample material was milled wheat grains of different cultivars. They were all identically prepared, thus the level of processing was not a factor affecting the results.

6.4 Future research

This study was qualitative by nature and an important aim was to screen extensive spectrum of cultivars and establish the method for the wheat prolamin extraction and separation. In future studies the aim should be in the quantitative approach. With further studies the accuracy of C-hordein reference material can be confirmed. In this study, the gliadin subgroups were not isolated and thus investigated separately in sandwich ELISA with the gliadin and C-hordein reference materials. This could be interesting to see in future studies in order to confirm if gliadin subunits from different cultivars would react similarly in spite of the different RP-HPLC patterns. Based on previous studies, it was expected that the ω-gliadin content would have been linked to a response in ELISA. In addition re-chromatography of the isolated prolamin subgroups could have been done in order to confirm the identity of each subgroup and to see if there would have been impurities visible.

As the technology of gluten quantification is getting more advanced we are able to detect even the smallest quantities of gluten. At the same time the accuracy of the method remains controversial. A way to improve ELISA method could be to standardize the current different kits with variety of cocktail-solutions, incubation times and temperatures, to one uniform extraction method. Reducing agents used in the extraction might interfere in the estimation of gliadin content, since they alter antibody-antigen binding (Doña et al. 2008). The variation of test results between different ELISA kits suggests that different protocols result in different results. This is of course challenging, because we are dealing with sample materials of different composition and processing history.

One of the reasons for inaccurate results is the current reference material, which needs a conversion factor to be used with, and therefore gives only an estimation of gluten quantity. The use of the factor two brings uncertainty to the analysis since also glutenins are extracted with aqueous alcohol solutions, as in this study, and R5 antibody does recognize glutenins in addition to gliadins (Kanerva et al. 2011). By developing a reference material that would by itself, without any conversion factors, give accurate results for all kinds of sample materials would surely be an aim for the future. However, as sample materials are diverse in their composition, it is not an easy task to find one reference material that would give accurate results for all of them. One solution could be to develop different materials for different grains. Based on the results of this study and earlier studies, C-hordein is promising reference material for the gluten quantification of both wheat and barley. How it would work with rye gluten quantification could be interesting to see. Between barley, wheat and rye, ω-gliadins show clear homology (Shewry et al. 1984). This would suggest that C-hordein could be used as reference material for rye too.

Finally, the method should strive to measure only the toxic compounds. In a previous study it was found that 50% of the coeliac disease patients did not respond to α-gliadin, but instead to a diverse set of gliadin and glutenin peptides (Vader et al. 2002). This proves that there is a wide variability in the recognition of these proteins among coeliac patients. It makes the gluten quantification method development challenging. As we are quantifying complex proteins, it needs to be carefully decided, what components of it is crucial to quantify.

7 CONCLUSIONS

In this study the aim was to develop a barley C-hordein reference material for wheat gluten quantification. The use of the current gliadin reference material has proven to result in inaccuracy of the results. It distorts the gluten content in the sample, because with it, a conversion factor is used to convert the gliadin content to gluten content. In other words, the resulted gluten quantity is only an estimate. The current reference material cannot cover the needs of variety of sample materials, which have different prolamin compositions. Additional problem is that it is not reproducible, thus it cannot keep up with the grains that undergo changes throughout years.

Several wheat cultivars from different countries were analysed in order to see the rate of variation they have in their prolamin composition. The hypothesis was that there would be great variation between wheat cultivars and that the current gliadin reference material would not be suitable to represent all of them in wheat gluten quantification. It was hypothesized that barley C-hordein would be able to represent the selected cultivars, since previous studies suggested that.

The method to separate gliadin- and glutenin subgroups in RP-HPLC was optimized (solvent to extract gliadin and glutenin, temperature, injection volume, gradient). The selected wheat cultivars were categorized into four groups based on similarities on their HPLC-patterns. For cv. Crusoe the ω-, α/β- and γ-gliadins and HMW- and LMW-glutenins were identified, although the silver staining of the SDS-PAGE gels was poor. The peaks were manually integrated and the proportion of ω-gliadin in total gliadin was calculated. Between the cultivars, ω-gliadin ranged from 0.8 to 14.1%, whereas for PWG-gliadin this has been reported to be 7.7%.

The whole gluten of different cultivars was isolated and investigated against R5 antibody in sandwich ELISA with gliadin and C-hordein standards. Km-values were calculated to compare the reactivity of the cultivars and the standards in the assay. The reactivity in ELISA could not be linked to ω-gliadin content of cultivar, although previous studies suggested that. In terms of similar reactivity in ELISA, 20% C-hordein was found to be the most suitable reference material for the selected wheat cultivars. The advantage of C-hordein standard is that the concentration and thus reactivity can be adjusted to match the sample materials with different prolamin profiles. Unlike

with current gliadin reference material, it can be used without any conversion factors, which improves the method accuracy.

Based on the results of this study C-hordein is a promising reference material for wheat gluten quantification. When adjusted with BSA in different ratios it could resolve the problem that the current reference material has with inaccuracy of the results. The 20 % C-hordein was found to be closest of the selected cultivars in terms of reactivity in the ELISA assay. The gliadin reference material was quite close to the reactivity of the selected cultivars too, but it has to be taken into account that no conversion factors was used in this study. With this model an average total gluten reactivity was able to be calculated for the selected wheat cultivars.

The measurement of gluten is a complex issue, as the components involved are complicated and their composition is different depending on the cultivar, country of origin and growing conditions. The results of this study together with previous studies highlight the need for new reference materials for wheat gluten quantification, as it is one of the weaknesses of the current method. The low limits of gluten accepted in gluten-free foods require high accuracy from the analytical methods.

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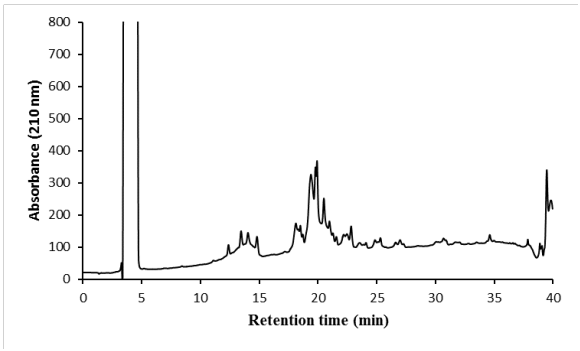
APPENDICES

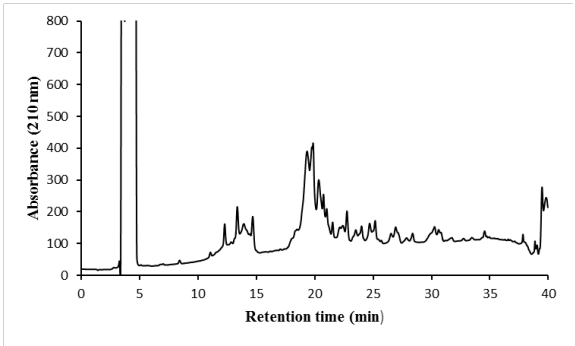
Appendix 1. Reverse-phase liquid chromatograms of glutenin extracts from selected wheat cultivars

Cultivars Apache, Britannia, Brons, Claire, Crusoe, Hereford, Revelation, Zulu, Julius (Germany), Gregory, Lancer, Lili, Mace, Siskin, Spitfire, Suntop were separated on a C8 column (50°C).

Apache



Britannia



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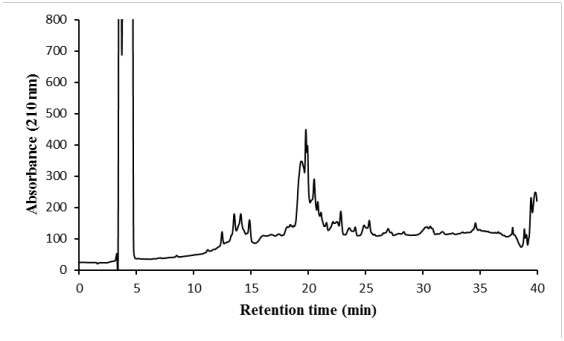
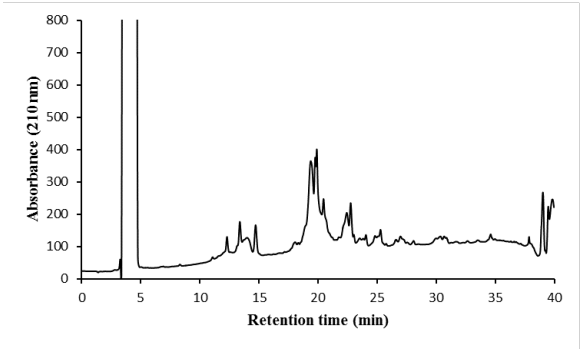
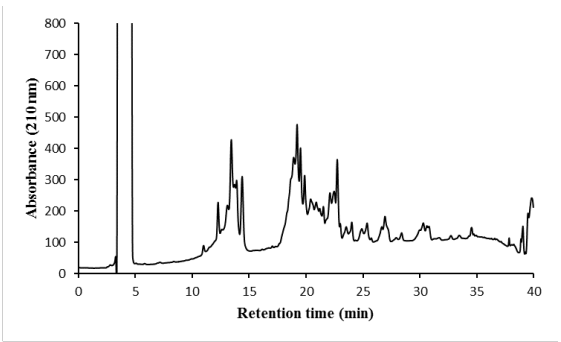


Claire



Crusoe



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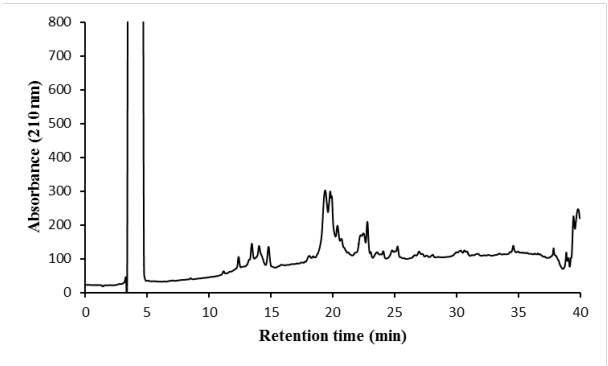
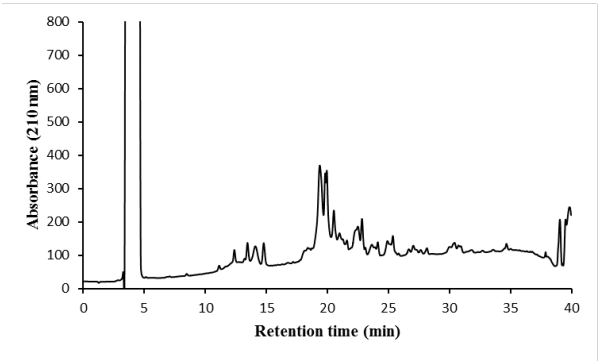
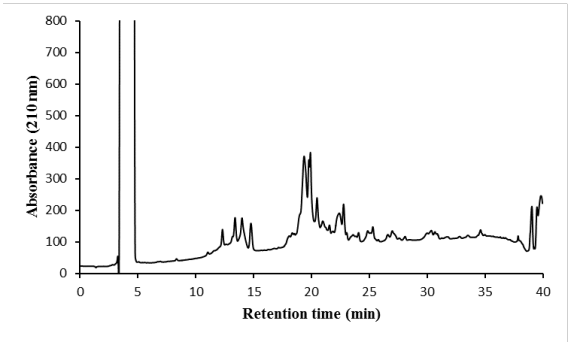


Revelation



Zulu



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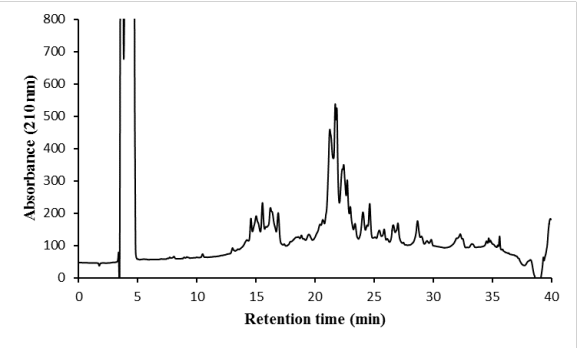
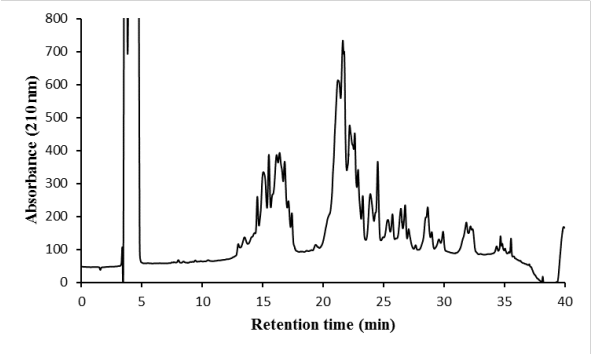
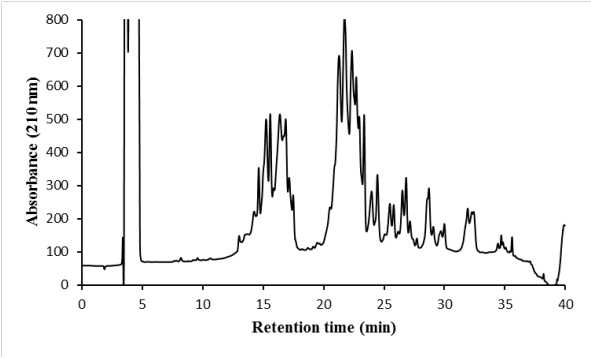


Gregory



Lancer



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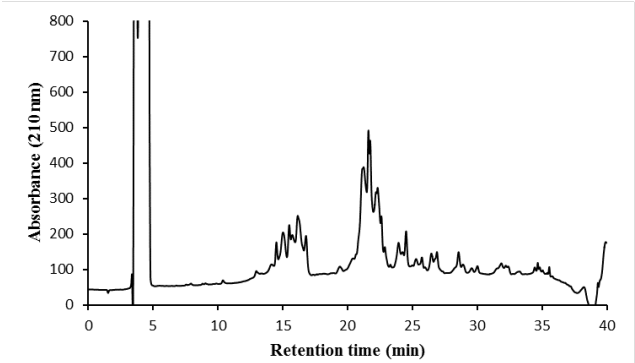

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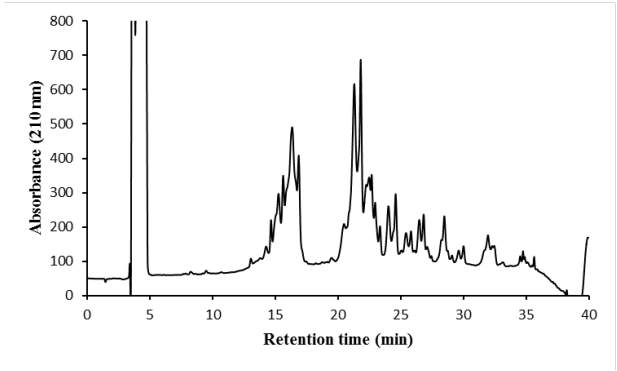

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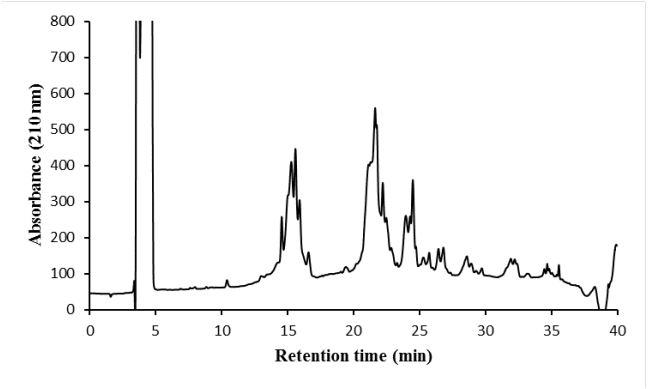

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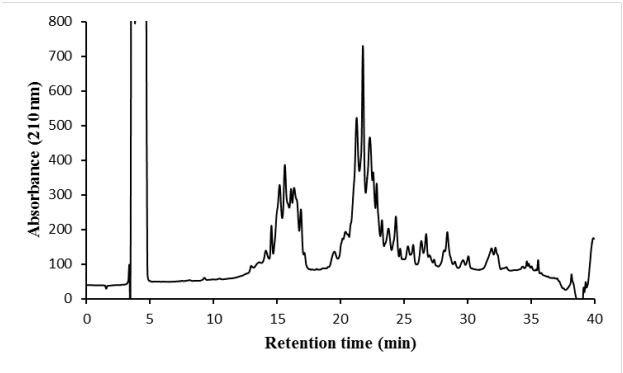

Suntop